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Early Blood Leucocyte Changes in Mice and Guinea Pigs Following X-Irradiation and Stress Caused by Operative Manipulations

By

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Abstract

Forssberg, A., B. Tribukait and K.-J. Vikterlöf. *Early blood leucocyte changes in mice and guinea pigs following X-irradiation and stress caused by operative manipulations.* Acta physiol. scand. 1961. *52.* 1—7. — Rapid shifts in the relative frequencies of the white cells of animals may occur following injection *e. g.* of toxins or other agents, radiation, but even mechanical manipulations (Jacobson 1954, Craddock 1960). The present work demonstrates the complexity of the reactions and the species differences between mice and guinea pigs following X-radiation and the manipulations (sham irradiation). A detailed survey (Table I—III) shows, that in mice eosinophils decrease following sham and X-radiation, that monocytes fall significantly by sham treatment but not furtheron after radiation, that rods increase continuously in number by application of sham treatment and X-rays. In these and other respects guinea pigs show a different behaviour, bearing witness to the multiformity of the reactions.

Leucocytes in general are apt to be released into the circulation from myeloid marrow at short notice. The capillary beds of various tissues seems to afford another reservoir (Harris 1959, Osgood 1954). The highly differentiated mode of reaction of the various leucocytes and the species differences is however not easily accounted for on the notion of a simple mechanism regulating the flow to and from the circulating blood.

The habitual action of ionizing radiation on the white cell picture of the circulating blood is that of causing a general concentration fall, which becomes manifest some 6—8 hours post-irradiation. The magnitude and duration of this decrease is dose dependant and may serve as an indication of a radiation influence of the organism in practice. Foregoing this stage a period of very rapid concentration changes during the first hours was noticed by many workers, although detailed analyses do not seem to have been performed.

On the other hand the white cells are very motile and a flow to and from the circulation is caused by a variety of measures including injections of chemicals, hormones among others. It may well be that the irradiation effects are mediated through release of some such substances. The complexity of the early white cell reactions was realised from our preliminary trials, and particularly the species differences between mice and guinea pigs. This induced us to a general survey of the early radiation reactions, including also the simultaneous stress effects caused by the mechnical manipulations of the animals.

Material and methods

Male guinea pigs were obtained from a commercial farm. They were caged for several days at the institute before the experiments. The weight of the animals varied between 150—250 g but within each series of experiments only within $\pm$ 10 g from the average weight. The mice were of our own heterozygote breed, weight 23—26 g in the main experiments. They were randomized on irradiation experiments and control tests as to cancel possible sex differences in reaction.

Irradiations were given at 250 kV and 15 mA, HVL = 1.6 mm Cu. The X-ray doses were in each instance measured during the exposures. In the case of guinea pigs the total dose was delivered in two sets from the distal sides and a third set from above in order to ensure uniform dose distribution. The mice were irradiated in a single delivery. Dose variations were obtained through distance and irradiation time variations. The irradiation time did in no case exceed 5 min.

Preliminary trials indicated that mice, contrary to guinea pigs, were very sensitive to the mechanical manipulation of bringing the animals into lucite boxes and giving a sham irradiation. Furthermore, an interaction between this stress effect and X-irradiation occurred, which was differently expressed in the various white cells and also showed species differences.

It was therefore found to be of interest to study the combined effect of manipulation and irradiation. A control group of directly from their permanent cages sacrificed animals was also included in the material as well as a third group of mice which were cautiously transported in their permanent cages to the place for irradiation. The reason for the latter procedure was that local conditions necessitated a transport from the animal room to another building for irradiation. An agitation of the animals was therefore unavoidable and the obvious procedure was to visualize experimentally the influence of each step of treatment.

In one series of experiments mice were irradiated under pentobarbitone ("Nembutal") anesthesia. They were given 2 mg pentobarbitone *i. p.* per 20 g weight before irradiation.

For blood analyses the guinea pigs were decapitated in a stage of slight ether anesthesia, whereas the mice were directly decapitated. Total leucocytes per mm^3 were counted in a Bürker chamber (mean of duplicate counts). Differential counts on Pappenheim

Table I. Differential counts of mice leucocytes under varying experimental conditions; animals sacrificed directly taken from their cages (1); subjected to manipulations in connection with irradiation (2); given 205 r and 595 r (3, 4); given pentobarbitone injection and irradiation in anesthesia as compared to directly sacrificed mice (5); animals cautiously transported in their permanent cages (perm.) and given 185 r as compared to directly sacrificed mice (6)

n = number of animals in each series.

Series	n	tot. leuco.	rods	segm.	eos.	mono.	lympho.
1 direct.....	29	7,202	6	738	70	135	6,253
2 sham	22	2,675	19	595	18	46	1,997
3 205 r	27	2,689	42	897	12	62	1,676
4 595 r	10	2,966	69	1,371	7	66	1,453
5 direct.....	8	8,462	8	743	52	127	7,532
pentob.	7	7,190	33	2,926	36	166	4,029
pentob. + 185 r	8	4,816	13	2,445	23	83	2,252
6 direct.....	8	6,516	—	626	43	134	5,713
perm.....	8	4,927	2	528	48	56	4,293
perm. 185 r ..	8	3,795	2	690	26	56	3,023

Table II. Differential counts of guinea pigs leucocytes. Experimental conditions in relevant respects similar to table I

Series	n	tot. leuco.	rods	segm.	eos.	mono.	lympho.
direct	10	4,439	14	1,123	21	102	3,166
sham	29	3,400	9	843	13	118	2,404
5r.....	10	3,374	15	1,115	32	139	2,062
24.....	10	5,832	11	3,112	34	253	2,388
50.....	11	7,863	26	5,054	29	444	2,247
200	15	5,087	97	3,395	44	273	1,257
400	15	3,106	169	2,214	23	221	465

stained samples were performed on 300 cells. Rod forms, segmented cells, eosinophils, basophils, monocytes and lymphocytes were scored. Basophils, which occurred in low concentrations and very irregularly in guinea pigs and are practically absent in mice, have not been tabulated.

Results

Earlier work has demonstrated that the peripheral leucocyte concentration increases during the first post-irradiation hours. Provided the doses are well below sublethal level, normal or nearly normal, values appear again after about 6 hours. A secondary rise is often seen at a later stage. Detailed analyses of the very early steps of this reaction does not seem to have been undertaken.

Table III. Significance of reactions of mice and guinea pigs towards sham treatment and X-radiation. P-values > 0.05 are not recorded

Measured	Mice	Guinea pigs
sham effects, decrease of:		
eos.	< 0.001	–
mono.	< 0.001	–
lympho.	< 0.001	–
increase of: rods	< 0.02 > 0.01	–
X-ray effects, increase of:		
rods	< 0.001	< 0.001
segm.	0.001	< 0.001
eos.	–	< 0.001
mono.	–	< 0.001
decrease of: eos.	< 0.001	–

Our preliminary trials indicated that the divergent reactions of the various leucocytes and the species differences were most pronounced 2—4 hours after the X-ray dose and about equally well expressed. Thus, in the main experiments irradiated and sham treated animals were sacrificed 2 and 4 hours after the treatment and the results were pooled in the tables.

Mice. The data are summarized in Table I and II and the significance of some main results, particularly in regard to the species differences, in Table III. Data with P-values > 0.05 have not been considered in the discussion and in Table III.

Comparing the two groups "direct" and "sham" (series 1 and 2) it appears that the manipulations concomitant to the preparation for irradiation causes a very pronounced effect which is most impressive in lymphocytes. The fall of lymphocytes accounts mainly for the reaction of circulating total leucocytes. It should be noted that the lymphocytes proved to be the only cell group which by all treatments decreased in number, mice and guinea pigs alike, whereas varying behaviour characterized all other cell forms. Sham treatment of mice increased rods but decreased eosinophils significantly. Application of X-rays of the order of 200 r (3) did not cause distinct effects, but when increasing the dose to about 600 r (4) a trend becomes obvious, namely that rods increase and eosinophils decrease continuously by application of irradiation on top of the sham manipulation. The statistical significance of both these trends was proved by the method of correlation of ranks (LEVY and PREIDEL 1959). It may be noted that monocytes do not react towards irradiation and that a slight increase of segmented is barely perceptible after the higher dose.

Apart from these series two minor experiments were performed, in the first of which we ventured an attempt to reduce the sham effects by pentobarbitone

injection prior to irradiation series (5). This failed, and besides a significant increase of rods, which also occurs in the sham group, a significant outflow of segmented cells was also found. There is a general trend of a decrease of all leucocytes by irradiation under anesthesia although not statistically significant in this small series. Finally, the possibility was considered that a cautious transport of the animals may reduce the stress effect (series 6). The counts indicate a considerable reduction of the manipulation effects, but a trend similar to that found in the sham series (2) is still obvious.

The possibility that younger mice, weight 9—10 g may react in a more pronounced manner was tried in a single experiment of one group of controls and a group given 200 r and analyzed 2 hours post-irradiation. The reaction of these young mice was, however, entirely similar to that of full grown animals.

Guinea pigs. The material is rather limited and does not allow for definite conclusions in all instances. However, it is safe to state that the guinea pigs differ in several respects, both qualitatively and quantitatively from mice. (Table II and III.)

In the first place the manipulations in connection with the irradiations did not cause such marked effects as by mice. The trend of a slight fall of all leucocyte cells except for monocytes is not significant in the present material. Studies of the X-ray effects were performed over a wider range of doses since guinea pigs are on the whole more radiosensitive than mice and show definite effects at least down to doses of the order of 25 r. The X-ray reactions of rods is in common with mice; moreover the tendency of a lymphocyte decrease, which is suggested in the mice experiments, becomes higly significant in guinea pigs following doses of 200—400 r. In other respects the reaction of guinea pigs differ from mice. There occurs a radiation induced increase of eosinophils, the dose dependence of which is not quite clear. An increase of segmented cells and monocytes in the irradiated animals is also highly significant. The data suggest however, that the X-ray effect may reach a maximum at doses somewhere round 50 r whereas higher doses cause another, superimposed, effect of diminishing the outflow of leucocytes.

Discussion

A diversity of reactions of the white blood cells in regard to the early release to, and disappearance from, the circulation has been found in the two species. Differential counts at such early stages are rather scarce in the literature. Some data are however provided by HULSE (1960) on rats given 100, 600 and 5,000 r. This author irradiated the animals in their ordinary cages, thus with a minimum of disturbances, and his controls nearly conform to our group "direct". As in our mice material radiation caused a decrease 4 hours after the dose of eosinophils; similarly monocytes showed no early influence and rods and segmented cells increased, although only after 600 and 5,000 r. There occurred in

rats also a considerable decrease of lymphocytes already after 100 r. This finding sheds some light on the reactions of lymphocytes in our mice material. Evidently the manipulations *per se* depleted the lymphocytes so that no further reduction by irradiation is achievable.

The rapid increase of circulating leucocytes seems partly to be due to a rapid mobilization of mature, or nearly mature, cells from mother tissues, preferably the marrow. HARRIS (1960) made studies of bone marrow and blood from irradiated guinea pigs. It appeared that at least segmented cells are discharged from the marrow in amounts which roughly accounts for the early increase of this cell group in the blood. Detailed analyses in regard to other cells and the dose dependence were however not performed.

The bone marrow on the other hand acts as a depot for lymphocytes which leave the circulation (HARRIS 1959 and YOFFEY 1960). There seems to be a reversed reaction in the sence that when granulocytes leave the marrow, lymphocytes are taken up. The general fall of circulating lymphocytes by all sorts of treatments in our case is likely explained in this way. Other tissues may also serve as a depot for segregated cells. Following treatment with adrenaline and histamine this was the case for lungs, spleen and intestines (AMBRUS and AMBRUS 1959).

Leucocytes are very motile cells which according to OSGOOD (1954) are moving to the sites where they are best needed. From quantitative counts of the distribution of total leucocytes in blood, bloodforming tissues and other tissues this author arrived at a figure of about 70 per cent in "other tissues" against only about 0.3 per cent in the blood. A vast reservoir is thus present here for rapid mobilization.

Another noteworthy feature is the complex relationship of sham treatment and irradiation, which may act synergistically or independantly of each other. The fact that radiation sensitivity and manipulation sensitivity do not always run parallel was clearly demonstrated in a work by ALBAUM (1960) on the reactions of whole body X-rayed rabbits serum enzymes. Early concentration changes occurred in the serum and from 14 enzymes assayed no less than 11 showed sham effects. Some of these showed additional X-ray effects, whereas others were unchanged by the irradiation. There were also enzymes which neither reacted towards irradiation nor towards sham manipulations. A wide reaction spectrum, similar to that of cells, is consequently also to be found on the molecular level. Since many of the enzymes which displayed different reaction had about the same molecular weight the particle size as such is not a decisive factor in the mechanism of controlling flow to and from the circulation.

Perhaps the most reasonable hypothesis is that the rapid and differentiated shifts of the localization of the various leucocytes is mediated by hormonal action or by the release of substances like histamine. An early rise of circulating total leucocytes is *e. g.* caused by adrenaline injection in humans. (LUCIA, LEONARD and FALCONER 1937). In dogs early eosinopenia occurred after the

injection of ACTH, adrenaline or histamine (LAST *et al.* 1950). Numerous other observations demonstrate similar actions on the white cell distribution.

That hormones may interfere with irradiation effects in a complex way on the chemical level has been visualized in other connections. Adrenaline and ACTH was, for example, found to change the rate of resorption and the exhalation of $^{14}CO_2$ from injected $NaH^{14}CO_3$ in mice (FORSSBERG and HEVESY 1955).

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Histamine Release Elicited by Extracts from Ascaris Suis — Influence of Oxygen Lack and Glucose

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Abstract

DIAMANT, B. *Histamine release elicited by extracts from Ascaris suis — influence of oxygen lack and glucose.* Acta physiol. scand. 1961. *52.* 8—22. — In guinea pigs intraperitoneal injection of *Ascaris* extract in doses effective in rats neither released histamine into the peritoneal fluid, nor "disrupted" mast cells in the mesentery. *In vitro* studies on *Ascaris*-induced histamine release from rat lung tissue showed the release to be dependant on pH, incubation time and concentration of extract. Under oxygen lack, the histamine release by *Ascaris* extract diminished to about 9 % of that in oxygen, but increased in the presence of glucose to about 80 % of the values found in the presence of both oxygen and glucose (5.6 mmole/l). With oxygen alone 60 % of this value was released. The enhancement of histamine release caused by glucose in nitrogen as well as oxygen suggests that it is dependant on enzymatic processes yielding high energy compounds.

During the past decades various theories have been presented concerning the mechanism of histamine release and mast cell "disruption". Several of the theories maintain that enzymatic processes are involved in these reactions. Thus, PARROT (1942), MONGAR and SCHILD (1957), CHAKRAVARTY (1959) and DIAMANT and UVNÄS (to be published) have shown that oxygen lack inhibits the histamine release as well as the mast cell "disruption". Further evidence has been presented by JUNQUEIRA and BEIGUELMAN (1955), MONGAR and SCHILD (1957), HÖGBERG and UVNÄS (1958, 1960), MOUSSATCHÉ and

PROVOUST-DANON (1958), CHAKRAVARTY (1959), UVNÄS and THON (1959, 1960) and others, who have shown that histamine release and mast cell "disruption" depend on pH and temperature and that various enzyme inhibitors act as blocking agents. These investigations concern the action of antigen on sensitized tissues and synthetic histamine liberators on non-sensitized tissues as well as on isolated mast cells *in vitro*. Compound 48/80 has been investigated far more extensively than other synthetic histamine liberators.

In addition to the synthetic histamine liberators there are several of biologic origin. A very active liberator can be extracted from *Ascaris suis*, an eelworm occurring in the hog. ROCHA E SILVA and GRAÑA (1946) showed that *Ascaris* extracts, when injected into dogs, "produced histamine shock indistinguishable from anaphylactic shock". *Ascaris* extract has also been found to release histamine *in vitro* from perfused cat paws and to "disrupt" mast cells of rat mesentery *in vitro*, (HÖGBERG, THUFVESSON and UVNÄS 1956, HÖGBERG *et al.* 1957). It has been suggested that the mast cell "disruption" caused by *Ascaris* extract involves enzymatic processes (UVNÄS *et al.* 1960). Our knowledge of these enzymatic processes is still, however, imperfect, though the blocking of mast cell "disruption" by such metabolic inhibitors as dinitrophenol, thyroxine, cyanide etc. indicates that the "disruption" is an energy requiring process.

The present investigation mainly concerns the histamine releasing activity of *Ascaris* extracts, tested in vitro on rat lung tissue under various conditions. The aim was to evaluate the role of glucose in *in vitro* histamine releasing reactions, as judged from the effect of *Ascaris* extract on rat lung tissue under the influence of nitrogen and oxygen respectively.

Materials and methods

Ascaris Extract

The preparation of *Ascaris* extract has been reported elsewhere. (UVNÄS *et al.* 1960). The activity of an extract, which heated to 100° C for 20 min in 50 % ethanol, is fairly stable when stored at 4° C. The amounts of extract referred to are valid for a dialysed acid extract dissolved in an equal volume of ethanol, the dry weight of 1 ml of which amounted to 6—10 mg, depending on the stock solution used. Three different stock solutions were used in the present experiments and are referred to in the following as *Ascaris* extract I, II and III.

Prior to all experiments the required amount of *Ascaris*-ethanol solution was dried *in vacuo* to eliminate the alcohol. The dry extract was then dissolved in a buffered isotonic solution (see below) and the pH was adjusted to between 6.9 and 7.2. In the experiments with isolated rat lung tissue the concentration of the *Ascaris* extract prepared always amounted to 10 times of that finally wanted. In the experiments with intraperitoneal injection of *Ascaris* extract into rats and guinea pigs *in vivo*, the *Ascaris* extract was further diluted with buffered isotonic solution to the required concentration.

Buffered Isotonic Solution

A solution containing NaCl (154 mmole/1), KCl (2.7 mmole/1) and $CaCl_2$ (anhydrous, 0.9 mmole/1) buffered with Sörensen phosphate buffer (67 mmole/1) 10 % v/v was

used as incubation medium in the experiments with isolated rat lung tissue. The pH of the medium was kept between 6.9 and 7.2.

Intraperitoneal Injection of Ascaris Extract

10 ml *Ascaris* extract, treated as described above, was injected intraperitoneally into rats (weighing 150—200 g) and guinea pigs (weighing 350—400 g) of both sexes. 10 min after the injection the animals were anesthetized with ether and killed by exsanguination. The abdomen was opened and the intraperitoneal fluid removed by pipette. The fluid was then centrifuged (2,500—3,000 r. p. m. for 5 min) and stored at — 20° C until assayed for histamine content.

Three pieces were cut from different areas of the mesentery, and were fixed and stained in a water solution containing 4 % formaldehyde and 0.1 % toluidine blue (NORTON 1954). At microscopic examination of the mesenteric specimens, a mast cell was considered to be "disrupted" if 3 or more granules were detected outside the cell. A hundred cells were counted from each specimen from which the "disruption" was calculated as percentage.

Experiments with Isolated Rat Lung Tissue

The method used corresponds, in general, with CHAKRAVARTY's (1959) modification of UNGAR and PARROT's (1936) method. Male and female rats weighing 250—450 g were used. After lightly anesthetizing the rat with ether the thorax was opened and the heart rapidly excised. This procedure precluded aspiration of blood into the lungs. Following exsanguination, the lungs were removed from 3—5 animals and stored in the buffered isotonic solution. All visible bronchi were removed from the lobes. Each lung lobe was divided into 6—12 approximately equal parts, depending on the number of samples required for the experiment. To assure a more or less uniform distribution of the rat lungs, all samples were prepared so as to include a portion of each lung lobe. The samples were blotted dry with filter paper and weighed, each sample usually weighing 500—600 mg (in no instance less than 400 mg). The samples were cut into smaller pieces (1—2 mg) and carefully washed with buffered isotonic solution. They were finally incubated in small Erlenmeyer flasks and rocked in a Warburg apparatus at 37° C. The time required for preparation of the lung tissue samples prior to incubation was $1^1/_2$—3 hours.

The lung samples were uniformly incubated for 15 min in the incubation medium before addition of the *Ascaris* extract. In the experiments on the influence of oxygen and nitrogen on histamine release the gases were bubbled through the solutions via syringe needles during this 15-minute period. In order to avoid foaming when the *Ascaris* extract was added to the samples, the needles were withdrawn above the surface of the solutions for the rest of the experiment. In the experiments where the effects of glucose as well as magnesium chloride were investigated, these substances were present in the buffered isotonic solution during preparation and incubation of the lung tissue samples.

Unless otherwise stated the *Ascaris* extract was kept in contact with the lung samples for 20 min. The incubation fluid was then filtered through cotton wool. After centrifugation, the pH of the incubation fluid was determined electrometrically and the fluid was stored at — 20° C for subsequent histamine assay.

In all experiments the *Ascaris* extract, concentrated as described above, was added in a volume of 0.1 ml/100 mg lung tissue. The final volume of incubation fluid amounted to 1 ml/100 mg lung tissue. In the various experiments the final concentration of *Ascaris* extract amounted to 0.5 ml/ml incubation fluid if not otherwise stated.

For extraction of the histamine remaining in the lung samples after the incubation

Table I. Effect of intraperitoneally injected Ascaris extract I in rats and guinea pigs

Species	ml *Ascaris* extract per ml buffer solution injected intraperitoneally	"Disrupted" mesenteric mast cells, in %	Histamine base per ml peritoneal fluid, in μg
Rat	0.2	49	0.50
»	0.5	81	0.93
»	0.5	100	1.70
»	Control	0	< 0.01
Guinea pig.....	0.5	0	0.03
»	0.5	0	0.03
»	Control	0	< 0.01

10 ml injected into each animal. In controls buffered isotonic solution without *Ascaris* extract I was used.

1 ml of the buffered isotonic solution was added per 100 mg lung tissue. Extraction was done by heating the separate samples for 30 min at 100° C on a water bath. The fluids were then filtered through cotton wool, centrifuged and stored at — 20° C until assayed for histamine.

The total histamine content varied between 2.4—9.1 μg histamine base per g of lung tissue in the different experiments.

In order to verify that all the remaining histamine was extracted by the above procedure, an additional extraction on random samples was performed *ad modum* FELDBERG and TALESNIK (1953), *i.e.*, by boiling over an open flame for 3 min with 8 ml buffer solution and 2 ml N HCl per g of lung tissue. Following centrifugation and neutralization, the amount of histamine remaining was never more than 3 % of the total histamine content of the lung samples. This small amount was disregarded when computing the histamine release as per cent of the total histamine content.

In order to investigate if the extracted histamine was inactivated when exposed to 100° C for 30 min, identically treated samples were extracted for 5 and 30 min respectively, with and without addition of N HCl. Since in all cases the amount of histamine extracted was found to be the same, this could be precluded.

The variation of *Ascaris*-induced histamine release in duplicate samples amounted on an average to $\pm$ 1.4 % of the total histamine content (range $\pm$ 0.1—$\pm$ 3.0) as judged from 7 duplicate tests from different experiments where the histamine release varied between 30—40 % of the total histamine content.

Histamine Assay

Histamine was assayed on atropinized (1.5×10^{-6} M atropine sulphate) guinea pig ileum by comparing the contraction induced by a standard histamine dihydrochloride solution with at least 4 contractions of each experimental sample. The *Ascaris* extract in the concentrations used caused no contraction of guinea pig ileum and did not influence the histamine response. Mepyramine (10^{-6}—10^{-7} M) completely abolished the contractions produced by randomly selected experimental samples.

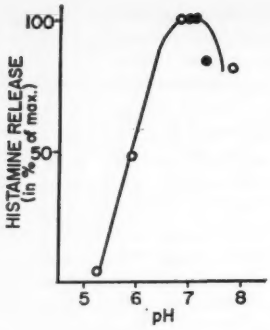

Fig. 1. Influence of pH on the histamine release caused by *Ascaris* extract from rat lung tissue. Values expressed in % of the maximum release in each experiment. The spontaneous release deducted from all values. ○—○—○—○ = *Ascaris* extract I (1 ml/ml incubation fluid). •—•—•—• = *Ascaris* extract III (1 ml/ml incubation fluid).

Results

Intraperitoneal Injection of Ascaris Extract in Rats and Guinea Pigs

Table I shows that *Ascaris* extract I, when injected intraperitoneally in rats, caused "disruption" of mesenteric mast cells as well as histamine release into the injected fluid. In guinea pigs, the injections caused only negligible histamine release, as compared with controls injected with buffered isotonic solution alone. "Disrupted" mast cells were in no case observed in guinea pig mesentery after intraperitoneal injection of *Ascaris* extract.

In Vitro Release of Histamine from Rat Lung Tissue Caused by Ascaris Extract

Influence of pH

The rat lung samples were prepared and incubated in incubation media of varying pH. Extreme pH values were produced by addition of N NaOH or N HCl. Since the lung tissue itself caused a shift towards a less acid or alkaline pH of the incubation media used, the pH values at either end of the graph in Fig. 1 were reached only after using solutions of pH 4.9 and pH 9.0 respectively. Solutions with the pH adjusted still more towards the alkaline side could not be tested; they converted the lung tissue into a slimy mass which was difficult to handle.

In the two experiments represented in Fig. 1, the final concentration of *Ascaris* extract was 1 ml/ml incubation fluid. The maximal histamine release, as seen in Fig. 1, was elicited by *Ascaris* extract when the pH of the fluid after incubation was 6.8—7.1. Thus, incubation media with pH 6.9—7.2 were used in the subsequent experiments.

Time Course of Histamine Release

In order to obtain a rough estimation of the time course of the histamine release, lung samples were incubated for different periods of time (1, 2, 4, 8, 16 and 32 min) following addition of the *Ascaris* extract. Fig. 2 shows the resulting values after *Ascaris* extract I 0.75 ml/ml incubation fluid.

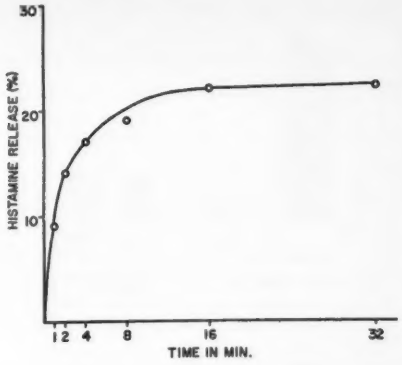

Fig. 2. Time course of *Ascaris*-induced histamine release from rat lung tissue. 0.75 ml *Ascaris* extract I per ml incubation fluid was used. Release values computed in % of the total histamine content. Spontaneous release deducted from all values.

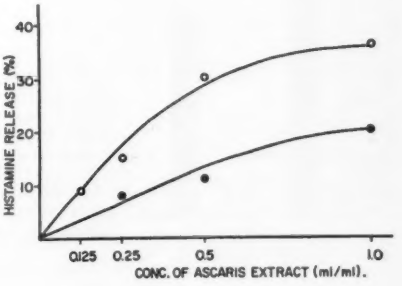

Fig. 3. Dose response. o—o—o—o = *Ascaris* extract II (stored 3 months at 4° C). •—•—•—• = *Ascaris* extract III (freshly prepared). The histamine release computed in % of the total histamine content. Spontaneous release deducted from all values.

About 50 % of the releasable histamine was liberated within 1 min after the addition of *Ascaris* extract. It seems clear that histamine release does not increase after 20 min, which has been the incubation time used in the present experiments.

Dose Response

Fig. 3 shows the histamine release caused by various concentrations of *Ascaris* extract II and *Ascaris* extract III. *Ascaris* extract II had been stored 3 months at 4° C while *Ascaris* extract III was freshly prepared. The distribution of the values are too large to permit detailed conclusions but the graph clearly shows the difference in histamine releasing activity of the 2 stock solutions of *Ascaris* extract tested.

In the experiments shown in the following figures, *Ascaris* extract III with a final concentration of 0.5 ml/ml incubation fluid was used throughout.

The Role of Oxygen

Fig. 4 shows that oxygen lack, produced by incubation of the lung samples under nitrogen, reduced the histamine release caused by *Ascaris* extract as

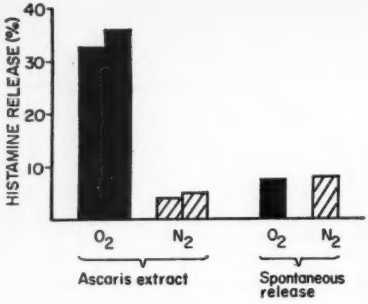

Fig. 4. Effect of nitrogen and oxygen on the histamine release elicited from rat lung tissue by *Ascaris* extract III (0.5 ml/ml incubation fluid). The variation of duplicate tests and the spontaneous release are shown. The spontaneous release is deducted from *Ascaris*-induced histamine release. All release values expressed in % of total histamine content.

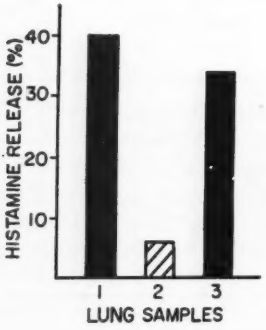

Fig. 5. Histamine releasing effect of *Ascaris* extract III (0.5 ml/ml incubation fluid) reincubated under oxygen (sample 3) after previous incubation under nitrogen (sample 2). Sample 1: Histamine release under oxygen without previous incubation of the extract under nitrogen. All values computed in % of the total histamine content. The spontaneous release is deducted from the release values of samples 1 and 2. Spontaneous release as well as the histamine release under nitrogen from sample 2 are deducted from the release value of sample 3.

compared with the histamine release under the influence of oxygen. The mean histamine release under nitrogen amounted to 5 % and under oxygen to 35 % of the total histamine content of the lung samples. This means that only 13 % of the histamine released under oxygen was liberated under nitrogen. It will also be noted that the spontaneous release with nitrogen approximated that with oxygen.

Table III A, on page 17, demonstrates the fall of histamine release under nitrogen compared with that under oxygen in 5 separate experiments. The histamine release under nitrogen averaged about 9 % of that under oxygen (range 2—14 %).

The possibility that the decreased amount of histamine found in the incubation fluids under nitrogen, was due either to an inactivation of the *Ascaris* extract or to a decomposition of the released histamine could be ruled out as shown in Fig. 5 and Table II. Fig. 5 shows that *Ascaris* extract, previously incubated with lung tissue under oxygen lack, when reincubated with a new lung sample under oxygen, released histamine in amounts comparable with those found in an oxygen control. This shows that the *Ascaris* extract was not inactivated under nitrogen when in contact with lung tissue. In addition,

Table II. Differences in histamine release and total histamine content between lung samples treated with oxygen and nitrogen respectively

Experimental conditions	Experiment no.									
	1		2		3		4		5	
Oxygen	2.2	(4.9)	2.4	(5.1)	1.9	(3.9)	2.3	(4.3)	2.1	(4.9)
Nitrogen.....	0.6	(5.1)	0.6	(5.3)	0.4	(3.8)	0.8	(4.2)	0.6	(4.8)
Difference	1.6	(−0.2)	1.8	(−0.2)	1.5	(0.1)	1.5	(0.1)	1.5	(0.1)

Histamine release values without parentheses; total histamine content in parentheses. All values are given as μg histamine base per g rat lung tissue. In each experiment 0.5 ml *Ascaris* extract II per ml incubation fluid was used. No correction has been made for the spontaneous release.

nitrogen bubbled through an *Ascaris* extract for 10 min did not change the histamine releasing activity.

Table II shows the difference in histamine release caused by *Ascaris* extract under oxygen and nitrogen respectively, as well as in the total histamine content of the corresponding lung samples. Since for each experiment the total histamine content shows only negligible differences as compared with the release values, the diminished histamine release found under nitrogen cannot have been due to decomposition of the histamine released into the incubation fluid.

Enhancement of Histamine Release under Nitrogen and Oxygen

MOUSSATCHÉ and PROVOUST-DANON (1957 A) reported that aerobic anaphylactic histamine release from guinea pig lung tissue *in vitro* was stimulated by the presence of metabolites of Krebs cycle, an effect which they attributed to an increase of high energy compounds. This effect could not be demonstrated, however, when the histamine release was caused by compound 48/80 (MOUSSATCHÉ and PROVOUST-DANON 1957 B).

It was, therefore, of interest to investigate the effect of glucose (yielding high energy compounds through its aerobic as well as anaerobic breakdown) and of magnesium ions (constituting a co-factor in several of these enzymatic processes) on the histamine release elicited by *Ascaris* extract from rat lung tissue *in vitro* under various conditions. In the presence of glucose (anhydrous, 5.6 mmole/l) and $MgCl_2$, $6H_2O$ (0.49 mmole/l) the inhibition of the histamine release was found to diminish markedly. Thus, under the influence of nitrogen the histamine release from 6 lung samples in the same experiment averaged 30 % of the total histamine content (range 27—33 %). From a 7th lung preparation under oxygen 34 % of the total histamine content was released in the presence of glucose and magnesium chloride in the above concentrations.

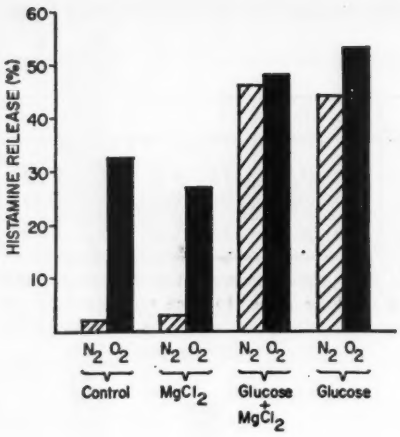

Fig. 6. Effect of glucose (5.6 mmole/l) and $MgCl_2$, $6H_2O$ (0.49 mmole/l) on the histamine release induced by *Ascaris* extract III (0.5 ml/ml incubation fluid) under oxygen and nitrogen. Control: Histamine release without influence of glucose or $MgCl_2$. Release values computed in % of total histamine content. The spontaneous release deducted from all values.

From this experiment, however, it was not possible to determine whether the presence of glucose or/and magnesium ions had caused the increased histamine release under nitrogen. A series of separate lung samples was, therefore, prepared in pairs with buffered isotonic solution containing glucose or/and magnesium chloride in the above concentrations. Lung samples treated with a solution containing no glucose or magnesium chloride were used as controls. One of each pair of lung preparations was incubated under nitrogen and the other under oxygen in the same solution previously used for the preparation.

It was found, as shown in Fig. 6, that under nitrogen the histamine release caused by *Ascaris* extract was enhanced solely because of the presence of glucose. The histamine release from the lung samples under oxygen was also found to increase in the presence of glucose. Thus, in this experiment, 53 % of the total histamine content was released from the lung sample treated with oxygen and glucose. With oxygen, but without glucose, the release decreased to 33 %. This means that about 60 % of the histamine release under oxygen in the presence of glucose occurred under oxygen alone. Magnesium chloride, in the concentration used had no demonstrable increasing effect on the histamine release under nitrogen or oxygen, either alone or in conjunction with glucose.

Table III B shows the histamine release caused by *Ascaris* extract under the influence of nitrogen in 4 different experiments with and without 5.6 mmole/l of glucose in the medium. Without glucose, as will be seen from the last column of Table III B, on the average only 2 % (range 0—5 %) was released as compared with the release when glucose was present. On the other hand, as shown in Table III C, representing 3 separate experiments, the histamine release under nitrogen in the presence of glucose averaged 82 % (range 75—88 %) of that under oxygen in the presence of glucose.

Table III. A. B. C. Influence of oxygen and nitrogen, with and without addition of glucose (5.6 mmole/l) on the in vitro histamine release elicited by Ascaris extract from rat lung tissue

A.

Expt. no.	Histamine release under the influence of		
	N_2 without glucose (in % of total histamine content)	O_2 without glucose (in % of total histamine content)	N_2 without glucose (in % of release under O_2)
1	4.5	34.4	13
2	6.0	44.9	13
3	0.3	14.8	2
4	5.6	40.4	14
5	1.9	34.8	5
			Mean: 9

B.

Expt. no.	Histamine release under the influence of		
	N_2 without glucose (in % of total histamine content)	N_2 with glucose (in % of total histamine content)	N_2 without glucose (in % of release with N_2 and glucose)
1	2.0	43.8	5
2	0.1	28.7	0
3	0.4	24.4	2
4	0.3	24.3	1
			Mean: 2

C.

Expt. no.	Histamine release under the influence of		
	N_2 with glucose (in % of total histamine content)	O_2 with glucose (in % of total histamine content)	N_2 with glucose (in % of release with O_2 and glucose)
1	30.0	34.2	88
2	30.1	40.3	75
3	43.8	53.6	82
			Mean: 82

The spontaneous release deducted from all values. Different stock solutions of *Ascaris* extract in amounts ranging from 0.5 to 1 ml/ml incubation fluid were used in the single experiments.

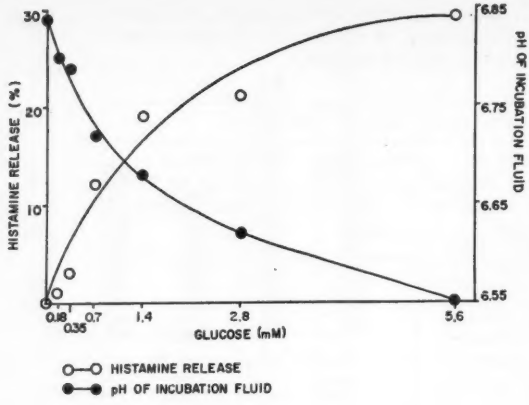

Fig. 7. Effect of various concentrations of glucose (geometrically decreasing from 5.6–0 mmole/l) on the histamine release under nitrogen elicited by *Ascaris* extract III 0.5 ml/ml incubation fluid) from rat lung tissue as well as on the pH of the incubation fluid after incubation. Release values computed in % of total histamine content. The spontaneous release deducted from all values. The pH of the incubation media with and without glucose was 6.90 before incubation.

Effect of Varying Concentrations of Glucose on Histamine Release under Nitrogen

The effect of different concentrations of glucose on the histamine release under nitrogen is shown in Fig. 7. The lung samples were prepared and incubated with media containing glucose in concentrations geometrically decreasing from 5.6 mmole/l. Before the addition to the lung samples the pH of the incubation medium was 6.90.

The histamine release caused by *Ascaris* extract under nitrogen was found to increase with increasing concentrations of glucose. Following incubation of the lung tissue, the pH of the incubation fluids fell as the glucose concentration increased.

Discussion

The species difference in histamine release as observed between rats and guinea pigs after intraperitoneal injection of *Ascaris* extract in this investigation is not surprising, since similar differences have been reported for other histamine releasing agents (Feldberg and Mongar 1954, Mongar 1955). The absence of "disrupted" mast cells in guinea pig mesentery does not, however, preclude the possibility that mast cells had been affected by the *Ascaris* extract. Boréus (1960) showed that in guinea pigs histamine liberating agents caused disappearance of the mast cells without the manifest "disruption" found in rats. Such a disappearance cannot be demonstrated with the method used in this investigation. The lack of detectable mast cell damage, however, parallels the negative finding of histamine release. Guinea pigs, therefore, are considered by the author to be insusceptible to *Ascaris* extract in the concentration used.

The histamine release from rat lung tissue elicited *in vitro* by *Ascaris* extract

is dependant on the pH of the incubation fluid, and maximal release values are obtained when the final pH is between 6.8 and 7.1. This accords with a previous investigation by UVNÄS *et al.* (1960) concerning the effect of *Ascaris* extract on mast cells *in situ*, for they noted a pH optimum around 7.0 for *Ascaris*-induced mast cell "disruption" in rat mesentery.

Judging from the time course of *Ascaris*-induced histamine release about 50 % of the releasable histamine was liberated into the incubation fluid within 1 min and the release was completed between 8 to 16 min after addition of *Ascaris* extract to the rat lung tissue. This is in accordance with the time course found by CHAKRAVARTY (1959) for anaphylactic histamine release from sensitized guinea pig lung tissue.

Different stock solutions of *Ascaris* extract when used under identical experimental conditions at the same concentration, show substantial variations in their histamine releasing capacity. This is attributed partially to unavoidable variations in the preparation procedure of the *Ascaris* extracts and partially to their inactivation during storage. Different degrees of inactivation are usually found after 2—3 months of storage with 50 % alcohol at 4° C. The inactivation occurs gradually. *Ascaris* extract used in experiments at varying intervals after preparation may thus give rise to variations in histamine release. Since, however, a single *Ascaris* extract was used in any given experiment, the comparisons presented refer to histamine release as elicited by the same stock solution.

The effect of oxygen lack on histamine release from lung tissue has previously been investigated by PARROT (1942), MONGAR and SCHILD (1957) and CHAKRAVARTY (1959). Using Tyrode solution as incubation medium, these authors showed that anaphylactic histamine release *in vitro* from guinea pig lung tissue requires oxygen. PARROT did not present figures but MONGAR and SCHILD reported an average of 32 % histamine release under oxygen lack (range 6—55 %) as compared with the release under air. CHAKRAVARTY found, with oxygen lack, a histamine release ranging between 14 and 29 % of the values found when oxygen was bubbled through the preparation during incubation. In rats, however, CHAKRAVARTY found no effect of oxygen lack on either anaphylactic or compound 48/80-induced histamine release from lung tissue incubated with Tyrode solution. MONGAR and SCHILD, furthermore, reported an increase under oxygen lack, of compound 48/80-induced histamine release from guinea pig lung tissue. Since guinea pig lung tissue is markedly insensitive to compound 48/80 and only responds when submitted to 100 to 1,000 times the concentration normally needed for histamine liberation in rat lung tissue (MONGAR and SCHILD 1957) the effect may be an unspecific one (BORÉUS 1960). In the present experiments without glucose, the *Ascaris*-induced histamine release from rat lung tissue *in vitro*, amounted, in oxygen lack, to only about 9 % of that found with oxygen. In earlier reports no information has been given concerning the presence of glucose in the Tyrode solution used. The fact that glucose greatly stimulates histamine liberation under oxygen lack, suggests

that previously reported variations in histamine release under oxygen lack may have been due, at least in part, to presence of glucose.

The acceleration of histamine release under oxygen lack in the presence of glucose was found to progress with increasing concentrations of glucose. At the same time the pH of the incubation fluid shifted more and more towards the acid side. Since *Ascaris*-induced histamine release was greatest at pH around 7.0, this shift towards the acid side cannot in itself be the cause of the increased histamine release.

The presence of 5.6 mmole/l of glucose was found to enhance histamine release elicited by *Ascaris* extract from rat lung tissue *in vitro* not only under nitrogen but also under oxygen. Thus far, the maximal histamine release has been found when the lung tissue was exposed to *Ascaris* extract under oxygen in the presence of glucose. Considering this release as 100 %, the release under the influence of nitrogen and glucose was somewhat less, amounting to about 80 %, with oxygen in the absence of glucose the release was reduced to about 60 %, with nitrogen in the absence of glucose less than 10 % was released. These figures must be regarded as approximate, and no statistical analysis has been undertaken. Similar results have been found, however, regarding the effect of glucose, oxygen and nitrogen, in respect to anaphylactic histamine release from rat lung as well as from guinea pig lung tissue, and also in respect to the release caused by compound 48/80 from rat lung tissue (DIAMANT, unpublished observations).

Anaphylactic histamine release from guinea pig lung tissue, according to MONGAR and SCHILD (1957) and CHAKRAVARTY (1959), involves enzymatic energy-requiring reactions. Their conclusions were drawn from the observation that lack of oxygen caused an inhibition of the histamine release *in vitro*. MOUSSATCHÉ and PROVOUST-DANON (1957 A) showed, as stated above, that several of the metabolites in Krebs cycle (succinate, a-ketoglutarate and acetate) had a stimulating effect on the aerobic anaphylactic histamine release from guinea pig lung tissue. This was attributed to an increase of high energy compounds arising from oxidation of the metabolites. The current observation of an increased histamine release produced by the addition of glucose, supports the view that enzymatic reactions are involved and that high energy compounds, deriving from the enzymatic breakdown of glucose, may be of major significance. It is apparent from the results that in the presence of glucose, even when oxygen is lacking, glycolysis may yield sufficient amounts of high energy compounds for histamine releasing reactions to occur.

In the present experiments, histamine was released from rat lung tissue under the influence of oxygen without addition of glucose, even though the experimental procedure involved treatment of the lung samples with glucose-free buffered isotonic solution for 1 $^1/_2$—3 hours prior to incubation. If, as suggested, high energy compounds are necessary for histamine release to occur, it may be inferred that glucose is retained in the lung tissue in amounts sufficing for

aerobic conditions. However, the possibility must not be discounted, as noted by BING (1954) and KREBS (1957), that in the presence of oxygen, high energy compounds may be formed from sources other than carbohydrates. On the other hand, in the absence of oxygen under corresponding experimental conditions, the glucose presumably retained in the lung tissue may be either depleted or insufficient to yield enough high energy compounds during the 15 minute period of nitrogen exposure preceding contact with the *Ascaris* extract. This would suggest that the diminished histamine release found under the influence of nitrogen may be due to lack of glucose and consequently of high energy compounds. This is further supported by the finding (DIAMANT, unpublished observations) that phlorizin under certain experimental conditions inhibits the stimulating effect of glucose on histamine release in the absence of oxygen.

High energy compounds are considered necessary for active transport mechanisms across cell membranes (LEHNINGER 1954). Such a mechanism might be involved in histamine releasing reactions in a way as yet undetermined. The final proof of an involvement of high energy compounds in histamine releasing reactions is, to judge from the relevant literature, still lacking, although such a dependency has been further supported by the finding, that agents which uncouple phosphorylation simultaneously produce inhibition of histamine release (MONGAR and SCHILD 1957, MOUSSATCHÉ and PROVOUST-DANON 1958, WESTERHOLM 1960, DIAMANT and UVNÄS to be published). From the present investigation it is, however, tempting to emphasize the correlation between the metabolic situation in various conditions with glucose, oxygen and nitrogen and the histamine release. Furthermore, investigations on mast cells from rat peritoneum *in situ* by DIAMANT and UVNÄS, (to be published) show that the "disruption" caused by compound 48/80 decreases under nitrogen and that this effect is counteracted by the presence of glucose.

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From the Institute of Pharmacology, University of Oslo, Norway

Note on the Effect of Increased NaCl-Concentration on the Neuromuscular Transmission

Does Desensitization to Acetylcholine Take Place during Tetanus?

By

GUNNAR LILLEHEIL and KNUT NAESS

Received 5 January 1961

Abstract

LILLEHEIL, G. and K. NAESS. *Note on the effect of increased NaCl-concentration on the neuromuscular transmission.* Acta physiol. scand. 1961. *52.* 23—31. — The possibility of desensitization of the motor end-plate to ACh during tetanus — as proposed by THESLEFF — and the possible role of this desensitization for production of Wedensky inhibition of the neuromuscular transmission, have been tested and are discussed.

As in THESLEFF's experiments increased concentration of NaCl has been used to obtain block of contraction and pure end-plate potentials. Mechanical registration of the contractions and extracellular recording of end-plate potentials were employed.

No signs of Wedensky-inhibition could be obtained by increasing the NaCl-concentration in the bath. Previous results indicate that the state of the motor end-plate after long sustained strong increase in the NaCl-concentrations must be rather unphysiological. The present results and also previous ones quoted in the discussion do not indicate any certain desensitization of the motor end-plate to ACh under physiological conditions. A mechanism of this type can probably not be of particular significance for the production of the very rapidly occurring Wedensky inhibition seen, for example, during curarization.

THESLEFF (1959) has recently published results obtained by use of increased concentration of NaCl (2.0—2.5 times the normal one) on the neuromuscular transmission in the rat phrenic nerve-diaphragm preparation. The purpose of

using this method was to reduce the mechanical movements of the muscle, making intracellular recording of end-plate potentials (e.p.p.) possible without using tubocurarine or magnesium. It had previously been demonstrated (HOWARTH 1958) that twitch responses are abolished by use of hypertonic solutions (concentrated Ringer's solution or solutions made hypertonic with sucrose).

FATT and KATZ (1952) examined the effect of sodium ions on the neuromuscular transmission. They confined the investigation to the effect of decreasing concentrations of sodium, but also made a few observations on the effect of increased concentrations of sodium and addition of sucrose, thereby differentiating between the osmotic and the specific ionic effects. Some of their results will be discussed in relation to those of THESLEFF and the present ones.

THESLEFF used intracellular microelectrodes and tetanic stimulation followed by electrophoretically applied acetylcholine (ACh) and concluded according to his experimental results that "desensitization of end-plate receptors is produced by the transmitter agent and can account for the decline in amplitude of the successive end-plate potentials. Furthermore, the desensitization process is likely to be at least partly responsible for the neuromuscular transmission failure known as the Wedensky inhibition."

We are at present engaged in investigation of the Wedensky-inhibition produced during curarization, which according to our results must be a primarily presynaptic effect produced by tubocurarine (LILLEHEIL and NAESS 1960 and 1961).

For our further work and a more detailed discussion of the possible presynaptic effect of tubocurarine, the present investigation and ensuing discussion have been necessary.

Methods

Rat phrenic nerve-diaphragm preparation were prepared according to BÜLBRING (1946). Isotonic contractions were recorded as usual on a kymograph and e.p.p. were recorded on submerged preparations with a chlorinated silver electrode with a diameter of 1 mm, isolated to the tip. The end-plate zone was located according to usual procedures (described in more detail by LILLEHEIL and NAESS 1961). Indirect supramaximal stimulation with impulses of 0.3 msec was used.

Oxygenated (+ 5 % CO_2) Tyrode's solution was used, and undissolved NaCl was added to the bath in an amount necessary to double the concentration of that salt. Decreased concentration of NaCl was used in a few experiments. Sucrose was added to make the solution isotonic. 1 VRU hyaluronidase (Invasin® "Lundbeck") per 100 ml was used in one experiment to make the connective tissue of the preparation more permeable for the salt solution and to obtain the neuromuscular block as rapidly as possible.

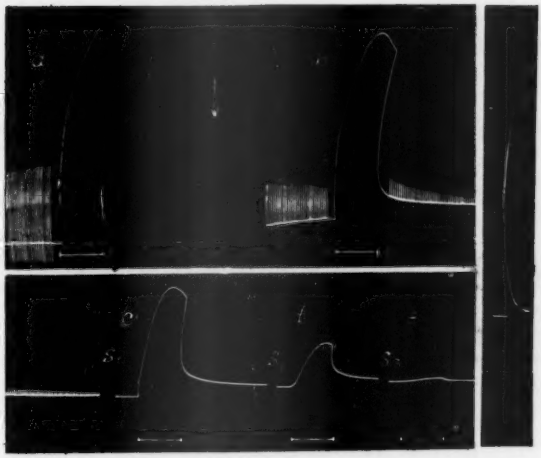

Fig. 1. Single and tetanic contractions of rat diaphragm preparation produced by indirect supramaximal stimulation of phrenic nerve with frequencies of 1/sec and 50/sec. a) Normal contractions. At arrow increase to twice the normal NaCl-conc. (2 × NaCl), b, c, d and e recorded 2, 7, 12 and 17 min afterwards. Drum stopped for 5 min at s_1, s_2 and s_3. At right a usual not fully developed, Wedensky-inhibited contraction produced by curarization.

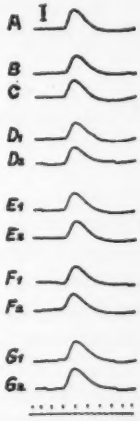

Fig. 2. Pure e.p.p. of rat phrenic nerve-diaphragm preparation recorded immediately after block had been obtained 60 min after change to 2 × NaCl. A, after single stimulation, B—G during tetanic stimulation with 50/sec. Time in sec for registration of potentials after start of stimulation: B : 0.5, C : 1, D_1—D_2 : 4, E_1—E_2 : 8, F_1—F_2 : 12, G_1—G_2 : 16. Hyaluronidase used to increase the penetration of NaCl.

Results

Usual mechanical recordings of single and tetanic contractions produced under increased concentrations of NaCl are presented in Fig. 1. It is quite obvious that no Wedensky-inhibition occur under these circumstances. Even with a frequency of 100/sec in a control experiment no sign of the slightest Wedensky-inhibition was observed. The reduced tetanic contractions have on

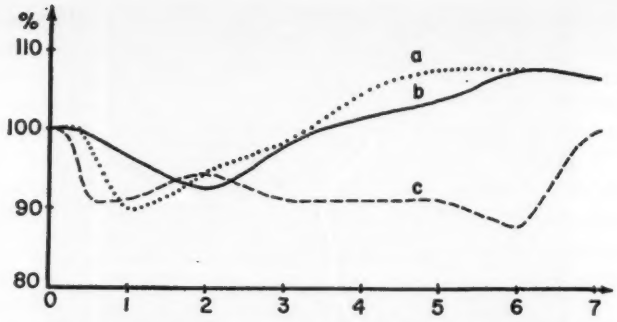

Fig. 3. Curves demonstrating variation of amplitude of e.p.p. during tetanic stimulation with 50/sec at different intervals after change to 2 × NaCl. Amplitude of single e.p.p. before tetanic ones = 100 %. Intervals after change of bath fluid: a: 45 min, b: 70 min, c: 60 min.

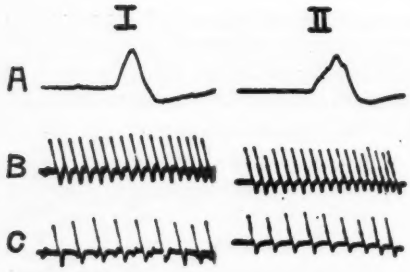

Fig. 4. Potentials recorded at the end-plate zone of rat diaphragm preparation, when the spike potentials are on the verge of disappearing. (E.p.p. approximately 90 % of total amplitude. Two different experiments (I and II) with recording 32 and 37 min after change to 2 × NaCl respectively. A: single potentials before tetanic volleys with 100/sec (B) and 50 sec (C).

the contrary quite the opposite appearance of what is expected of a Wedensky-inhibited contraction (which is, for comparison, added in Fig. 1). Even when the tetanic contraction is on the verge of disappearing (e), there is still a tendency to a slow increase of the very low amplitude during the period of stimulation.

Fig. 2 demonstrates the surprising constancy of the e.p.p. during tetanic stimulation, a result obtained in several experiments. The e.p.p. keep on a relatively stable level throughout the period of stimulation.

Fig. 3 demonstrates the approximately steady level of e.p.p. in three different experiments. A small reduction to about 90 % of the original values takes place during the first second of stimulation with 50/sec, but a compensating effect seems to be in action when the stimulation goes on. We dare not say that this development is a quite constant phenomenon, and it seems that the compensating process decreases when the excess of NaCl has worked for a longer time. We have also tried to measure the variation of e.p.p. before

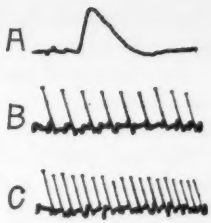

Fig. 5. E.p.p. of rat diaphragm preparation blocked by use of low sodium (appr. 1/3 of normal conc., sucrose added to compensate for loss of molarity). Recorded 20 min after change of fluid. A: single e.p.p. before tetanic volleys with 50/sec (B) and 100/sec (C).

a complete block could be obtained (with superimposed spike potential clearly distinguishable from e.p.p.) and we really found a well sustained increase of approximately 15 % of the original e.p.p. This result has, however, been omitted from Fig. 3, because movements of still contracting preparation may cause small irrelevant variation in the amplitude of e.p.p. The increase in e.p.p. is, however, in accordance with the slow increase in the amplitude of the contractions before complete block is obtained.

Fig. 4 demonstrates potentials obtained by stimulation with frequencies of 50 and 100/sec. shortly before pure e.p.p. was obtained. Only a very small fraction is not e.p.p., and no contraction occurs during tetanic stimulation at this stage of approximately complete block. We found a slight but readily observable increase of potentials in this introductory stage of stimulation when 50/sec was used and an approximately stable level with 100/sec. At a later stage of the NaCl action we usually found a small decrease of the e.p.p. just after the beginning of the stimulation, and our results are in this respect in conformity with those of THESLEFF. The decrease was, however, never of the magnitude seen at the stage of curarization when a Wedensky inhibition is well developed (LILLEHEIL and NAESS 1960 and 1961).

To complete the picture the possible change in the amplitude of the e.p.p. after reduction of NaCl have also been tested (Fig. 5). The stability of the e.p.p., especially in the first stage of a tetanus was again the most remarkable feature of this type of block, even if there was a slow decrease of the e.p.p. during a long sustained tetanus.

Discussion

Both mechanical recording and registration of e.p.p. demonstrate that no Wedensky inhibition is obtained by increased concentrations of NaCl. It seems, therefore, impossible to explain the mechanism behind any Wedensky inhibition by a hypothesis based on a decline of the e.p.p. during the influence of increased amounts of these ions. The reduction of e.p.p. is not at all a con-

stant phenomenon. If a reduction takes place, it develops very slowly and it is relatively small.

An increase of the e.p.p. can be obtained even in the first phase of stimulation, when the mechanism behind the very rapidly developing Wedensky inhibition — for example during curarization — must be at work.

HUTTER (1952) also failed to find any change of the ACh-sensitivity of the motor end-plate during a Wedensky inhibition produced by tubocurarine, and concluded that this phenomenon must be of pure presynaptic origin, a result which also conflicts with the hypothesis put forward by THESLEFF (1959). (See addendum.)

Previously described changes in the neuromuscular transmission produced by increased sodium or increased osmotic pressure

Increased concentrations of NaCl probably have two different effects on the processes invo ved in the neuromuscular transmission: i) the specific effects of the increased concentrations of the two ions, ii) an unspecific effect of the increased osmotic pressure. FATT and KATZ (1952) examined the effect of increased concentrations of sodium and of sucrose on the e.p.p. of frog and found two opposite effects. Sucrose decreased the e.p.p. but sodium itself produced a tendency to increase. The two effects may compensate for each other keeping the e.p.p. on a steady level, but the increasing effect of the sodium ion overcompensates for the depressive effect of the increased osmotic pressure when curarized preparations are used.

LILEY (1956) demonstrated a double effect of increased osmotic pressure produced by sucrose, *viz.* a significant increase of the frequency of the miniature potentials and at the same time a reduction of the e.p.p. was obtained (40 % with a 25 % increase in the osmotic pressure). An increase of the frequency of the miniature potentials is believed to be an indication of a tendency to increased release of ACh by nerve impulses (ECCLES, PIHL and LILEY 1959). Two different mechanisms which counteract each other seem, therefore, to be in action during increase of osmotic pressure, the postsynaptic decrease of the acetylcholine-sensitivity being the dominant one.

THESLEFF found no change of the amplitude of the miniature potentials in his experiments with increased NaCl-concentrations, a finding which was taken as a proof of an unchanged sensitivity of the end-plate. This may be in accordance with the results of FATT and KATZ, who have demonstrated that different mechanisms which compensate each other are at work under such circumstances. The unchanged ACh-sensitivity under the influence of increased NaCl-concentrations does, thus, not imply that the postsynaptic membrane is uninfluenced by the unphysiological condition produced by the high NaCl-concentration and reacts in a normal manner to different experi-

mental procedures — as for example — the combination of tetanic stimulation and ACh-application used by THESLEFF.

Different stages of effect of increased NaCl-concentration

The neuromuscular effects of small ions such as magnesium and calcium are rapidly produced, and it is also highly probable that the specific effects of sodium are produced as rapidly, while the effects which are due to the increased osmotic pressure must take some time to become fully established. It is obviously impossible to distinguish completely between these two actions in an experiment. It is, however, reasonable to presume that the specific effect of sodium is the predominant one in the first phase of action, while that of the changed osmotic pressure produces its strongest effect in a later stage. When both mechanisms of action have produced their full effect the preparation works under such unphysiological conditions, that deductions about physiological behaviour must be made with the utmost caution.

Result of present investigation in relation to previous results

In the first phase of the action of the increased NaCl-concentration we found a tendency to augmentation of the e.p.p., — and no decrease — even if stimulation with frequencies as high as 100/sec was used. The decrease of e.p.p. obtained during stimulation at a later stage was, furthermore, limited. The relative constancy of the amplitude of the e.p.p. during sustained tetanic stimulation was in our investigation the most remarkable feature of the NaCl-inhibited transmission. It is, therefore, not impossible that the results obtained by THESLEFF, *i. e.* the strongly reduced effect of electrophoretically applied ACh immediately after stimulation with tetanic bursts of moderate frequencies, may be due to the unphysiological condition of the preparation.

The mechanism behind the low sodium block will not be discussed in this paper. The reader is referred to the work of FATT and KATZ. The only point which it is necessary to keep in mind for this discussion is the stability of the e.p.p. also when the block has been obtained by this method. It is tempting to state that the consistant conclusion of our experiments is: The e.p.p. probably does not change even during the introductory stage of a tetanus under normal physiological conditions, when no distinct change can be observed immediately after attainment of pure measurable e.p.p. by either low or high sodium. The typical Wedensky inhibition, characterized by transformation of a tetanic contraction to a very shortlived twitch, is according to this conclusion probably not caused by any normally existing change of the output or sensitivity to ACh during the first phase of tetanus. This conclusion is also consistant with results obtained during the introductory stage of curarization, when the stability of the e.p.p. also was the relatively surprising experimental finding (LILLEHEIL and NAESS 1960 and 1961).

More direct evidence of the ACh-sensitivity of the motor end-plate has previously been obtained by LILEY (1956). This author has demonstrated that no change of the amplitude of the miniature potentials occurs during or immediately after a tetanus. This seems to be a direct proof of an unchanged sensitivity of the motor end-plate during tetanic stimulation. In this connection there is also reason to repeat the results of HUTTER, quoted in the beginning of the discussion: The acetylcholine sensitivity is not changed during a Wedensky inhibition, *i. e.* during a tetanic stimulation. It is still very difficult to examine e.p.p. changes during a tetanus in a mammalian preparation uninfluenced by drugs, toxins or ions. It is, however, tempting to look to the ganglionic transmission for comparison. ECCLES (1955) has recorded synaptic potentials by microelectrodes in ganglia of rabbits. The relative stability of these potentials, even at relatively higher frequencies (up to 40—50/sec) was remarkable in this investigation too. A further discussion of some of these and similar results will be taken up in our following work on the presynaptic effect of tubocurarine.

We would, however, at the end of this discussion emphasize that it is impossible by our experiments to prove that desensitization does not occur at all during a tetanus. It may, of course, be that development of desensitization is counteracted and compensated — or even overcompensated by a real increase of the release of ACh during the first phase of a tetanic stimulation. It may be that such a relatively small naturally occurring desensitization is aggravated under the conditions brought about by the increased NaCl-concentration and the hyperosmorality produced thereby. THESLEFF's results demonstrate quite clearly that a desensitization really exists under the experimental conditions of his experiments. The decrease of the e.p.p. produced by administration of ACh was, however, surprisingly strong compared with the often relatively small decline in the e.p.p. produced by indirect stimulation. This seems to imply that an increase in the ACh-release per impulse probable must take place during the introductory phase of tetanic stimulation. Other reports which will not be mentioned here, indicate that the activity in the presynaptic fibres combined with conduction or electronic spread of the action potential and the release of ACh produced thereby, is followed by a state of increased preparedness for releasing ACh (for references see ECCLES *et al.* 1959). Such a mechanism would produce a potentiation of ACh-release during the transmission of repetitive stimuli — a process eventually compensating for a possible desensitization. More indirect evidence seems for the present — as mentioned above — to contradict a process of desensitization during a short tetanus under physiological conditions. That a desensitization to ACh takes place during a more prolonged tetanic contraction has been demonstrated by KRNJEVIĆ and MILEDI (1958). The course of the release of ACh during tetanic stimulation will be more comprehensively discussed in our following paper on the presynaptic effect of tubocurarine.

Addendum

OTSUKA and ENDO (1960) have quite recently published results obtained in experiments with frogs to test the hypothesis of desensitization put forward by THESLEFF. They used intracellular recording of e.p.p. and electrophoretical application of ACh in curarized preparations. No sign of desensitization to ACh could be recorded after conditioning repetitive stimulation. OTSUKA and ENDO, therefore concluded that the sensitivity of the end-plate to ACh remains entirely unchanged even when the amplitude of end-plate potential is markedly decreased after repetitive stimulation. Their conclusion was, accordingly, in agreement with that of HUTTER (1952) quoted in our article: The Wedensky inhibition must be due to a pure presynaptic procedure. OTSUKA and ENDO mention that the difference between their results and conclusion and THESLEFF's might be due to use of different species, but HUTTER used a mammalian preparation.

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Acta physiol. scand. 1961. 52. 32—35

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Respiratory Response to Acute Exercise in Induced Metabolic Acidosis

By

H. E. Refsum

Received 6 January 1961.

Abstract

Refsum, H. E. *Respiratory response to acute exercise in induced metabolic acidosis.* Acta physiol. scand. 1961. *52.* 32—35. — Ventilation, carbon dioxide output and oxygen uptake were recorded before, during and after a work of 500 kgm in one minute — before and after inducing a marked metabolic acidosis by means of ammonium chloride. While the resting ventilation in the acidotic state was moderately increased, the ventilatory increase due to the exercise was extremely high. The excess carbon dioxide blown off simultaneously, however, was very moderate. The increases of the resting and working ventilations were due to increases in the tidal volumes only. The recovery times of carbon dioxide output and ventilation were markedly increased, while the recovery times of oxygen uptake showed no definite change. The described pattern of respiratory response to acute exercise is often found in patients with cardiac or cardiopulmonary disease.

Using a standard exercise test for evaluation of cardiorespiratory function (Erikson 1952, 1957), consisting of recording carbon dioxide output, oxygen uptake and ventilation in connection with the performance of 500 kgm work in one minute, Lange Andersen (1960) has found carbon dioxide recovery times of 2—5 min and ventilatory increases of 20—40 l (ATPS) in young, healthy men. Erikson (1960) has found that in severe cardiorespiratory disease the carbon dioxide recovery times may be increased up to 15—20 min, and Erikson and Müller (1960) have shown that abnormally high ventilatory in-

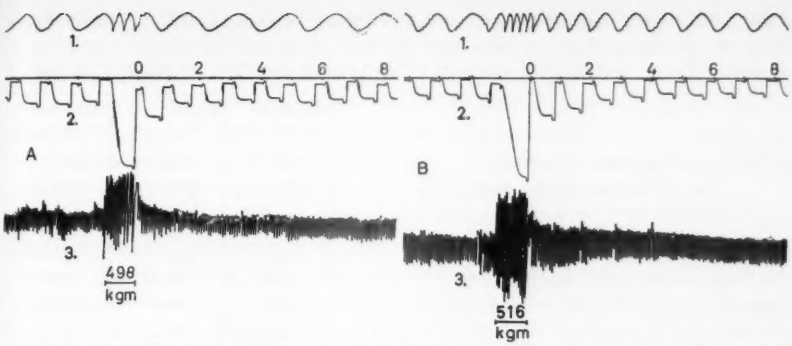

Fig. 1. Sections of the spirometer curves, recorded before intake of ammonium chloride (A) and after intake of 55 g ammonium chloride in the course of 48 hours (B). (1: Ventilation. 2: Carbon dioxide output. 3: Respiratory difference curve.)

creases, exceeding 60 l (ATPS) in patients with pulmonary disease, and 45 l in patients with no signs of pulmonary disease, strongly indicate cardiac disease, provided that no acidosis is present.

Earlier investigations have demonstrated the influence of experimentally induced metabolic acidosis on the ventilation at rest and during steady state work (Haldane 1921, Dennig *et al.* 1931, Nielsen 1936, and others). The purpose of this work has been to investigate the influence of induced metabolic acidosis on the respiratory response to acute exercise.

Methods and procedure

The standard exercise test was performed according to Erikson (1952, 1957), by recording the carbon dioxide output, oxygen uptake and pulmonary ventilation per minute during a resting period of 20 min, one minute exercise (performing 500 kgm on a bicycle ergometer), and the following 20 min of recovery and rest.

The recovery times for the carbon dioxide output, oxygen uptake and ventilation were defined as the times from the end of the exercise till the value of the actual parameter was less than 10 per cent above the mean resting value. The increases in carbon dioxide output, oxygen uptake and ventilation due to the exercise were determined as the differences between the total value of the actual parameter during a 10 min period — including the last pre-exercise minute, the exercise minute and the following 8 min — and the mean resting values for 10 min.

The exercise test was performed on 3 consecutive mornings by a healthy, well trained male subject (32 years, height 190 cm, weight 79.0 kg). Between the first and the second test 10 portions of 2.5 g ammonium chloride were taken *per os*; between the second and the third test additionally 30 g were taken in the same way. The total intake of ammonium chloride in the course of 48 hours was thus 55 g.

Before each exercise test a venous blood sample was taken, and the CO_2 combining power of plasma determined according to Van Slyke and Cullen (1917). Forty-five minutes after the last test an arterial blood sample was taken and the blood gas values determined (Refsum 1960).

Table I. Respiratory response to acute exercise in induced metabolic acidosis. (Control: Before intake of ammonium chloride. 24 hrs.: After intake of 25 g ammonium chloride in the course of 24 hours. 48 hrs.: After intake of additional 30 g ammonium chloride in the following 24 hours.)

	Control	24 hrs.	48 hrs.
Resting oxygen uptake, ml/min	283	274	276
» carbon dioxide output, ml/min	258	246	242
» ventilation, l/min	7.9	9.4	10.5
» respiratory rate	13.7	13.3	13.8
Work, kgm performed in one min	498	510	516
Net work efficiency, per cent	20.4	20.8	19.7
Increase in ventilation due to work, l	22.5	44.4	64.0
Working respiratory rate	16	16	17
Increase in carbon dioxide output due to work, l	1.25	1.55	1.65
Recovery time of carbon dioxide output, min	1	3	4
» » » oxygen uptake, min	1	2	2
» » » ventilation, min	1	3	5

All volumes are given at ATPS.

Results

On the first experimental day the CO_2 combining power of plasma was found to be 26.0 meq/l (*i. e.* quite normal), while it before the second test (after intake of 25 g ammonium chloride in the course of 24 hours) had decreased to 15.7 meq/l, and before the third exercise test (after an additional intake of 30 g during the following 24 hours) it was 13.5 meq/l. An arterial blood sample taken 45 min after the last exercise test showed CO_2 content (plasma): 11.5 meq/l, pH: 7.13, CO_2 tension: 32 mm Hg, hemoglobin oxygen saturation: 97.5 per cent. During the experiment the body weight changed from 79.0 kg on the first day to 77.0 kg on the second, and 76.0 kg on the third day.

Fig. 1 shows sections of the spirometer curves, recorded on the first day, before the intake of ammonium chloride, and on the third day, after intake of 55 g in the course of 48 hours. The data calculated from the three curves recorded are presented in Table I.

Comments

The resting oxygen uptake and carbon dioxide output, the work performed and the net work efficiency varied within relatively narrow limits in all tests. This indicates that the experimental conditions, as far as the exercise tests are concerned, must have been practically identical.

The intake of ammonium chloride led to a marked increase of the resting ventilation, the last value being 33 per cent higher than the control value; but all values were within the range for normal subjects. The ventilatory in-

crease due to the exercise increased from a low, normal value in the control test to a high, normal value on the first day with acidosis, and an abnormally high value on the second day, with severe acidosis; this value being approximately 185 per cent higher than the control value. It appears further that the respiratory rates, both for the resting periods and the exercise minutes, were the same in all three tests, showing that the increases in resting and working ventilation were the results of increases in the tidal volumes only.

While the resting values of carbon dioxide output were practically the same in all three tests, the increase in carbon dioxide output due to the exercise increased clearly during the acidosis, the last value being approximately 30 per cent higher than the control value. It appears, however, that the very high increases in the ventilation due to the exercise only led to a very moderate excess output of carbon dioxide, and consequently to a very low carbon dioxide concentration in the expired air (cp. ERIKSON 1957). The ventilation and carbon dioxide recovery times increased from low, normal values in the control test to high, normal values in the last test, while the oxygen recovery time showed no definite change.

It is seen that experimentally induced metabolic acidosis in a normal subject, without signs of cardiopulmonary or cardiac disease, can lead to the same respiratory response to brief, relatively moderate exercise as often found in patients with definite cardiac or cardiopulmonary disease (ERIKSON and MÜLLER 1960), *i. e.* with resting ventilation within the normal range, and abnormally high ventilatory increase due to the exercise, and increased carbon dioxide recovery time.

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Effect of Dietary Selenium Dioxide, Cystine, Ethoxyquin and Vitamin E on Lipid Autoxidation in Chick Tissues

By

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Abstract

BIERI, J. G., H. DAM, I. PRANGE and E. SØNDERGAARD, *Effect of dietary selenium dioxide, cystine, ethoxyquin and vitamin E on lipid autoxidation in chick tissues.* Acta physiol. scand. 1961. *52.* 36—43. — The ability of dietary selenium dioxide, L-cystine, ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) and dl-α-tocopheryl acetate to inhibit autoxidation in incubated tissue homogenates was studied using the thiobarbituric acid test. Ethoxyquin (0.1 %) was as effective a tissue antioxidant as was dl-α-tocopheryl acetate (0.01 %). SeO_2 (0.14, 0.46 and 1.4 ppm) significantly inhibited autoxidation in the liver, kidney and heart; the effect varied with different vitamin E-free diets. L-cystine (0.3 %) reduced autoxidation in muscle when fed in a casein-gelatine diet.

The autoxidation of tissue fatty acids which occurs when homogenates or cell particulate components are incubated in air can be greatly reduced if sufficient antioxidant, in the form of vitamin E, is fed to animals prior to the removal of tissues (TAPPEL and ZALKIN 1959, MAGHLIN et al. 1959, BIERI and ANDERSON 1960). In addition to this effect by vitamin E, it was found that under

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Table I. Composition of basal diets in percentages

	Diet no.		
	2,304	2,343	2,383
Torula yeast 3N[1]	60		
Soybean protein[2]		30	
Casein[3]			15
Gelatine	3		10
Salt mixture[4]	5.17	5.17	5.17
Vitamin mixture[4]	0.1	0.1	0.1
Choline chloride	0.2	0.2	0.2
Sucrose	31.53	63.53	68.53
Lard		1	1

The diets were supplemented with 1 mg Synkavit "Roche" (Di-calcium salt of 2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester) per 100 g. Vitamins A and D_3 were given as a solution prepared from crystalline vitamin A acetate (Roche), 1 g; crystalline vitamin D_3 (Roche), 0.0058 g; "Tween 80," 64 g; ethyl alcohol, 100 ml; and distilled water to a total volume of 330 ml. 0.1 ml twice a week per animal furnished 250 i. u. vitamin A and 20 i. u. vitamin D_3 per day.

[1] From Lake States Yeast Corporation, Rhinelander, Wisconsin, U. S. A.
[2] ADM Assay Protein C-1 from Archer-Daniels-Midland Company, Cincinnati, Ohio, U. S. A.
[3] "Dairinex," from A/S Dansk Mejeri Industri & Export Kompagni, Stege, Denmark.
[4] Dam and Søndergaard (1953).

some conditions the feeding of selenite to chicks significantly decreased lipid peroxidation in liver while the feeding of L-cystine decreased peroxidation in muscle (BIERI 1959). The present report extends these observations to other tissues when chicks are fed different types of vitamin E-free diets.

Experimental

Day-old New Hampshire and White Leghorn chicks were given a commercial starter ration for 6 days. They were then grouped and fed the experimental diets shown in Table I. The vitamin E-free basal diets differed primarily in their protein source. After 21 to 35 days on the experimental diets, the birds were sacrificed and the tissues removed. Testing of autoxidation of fatty acids was begun the day of sacrifice; however, for some tissues a storage at — 20° C for a period up to 74 days was required to finish all the determinations. We have observed (unpublished results) that frozen storage of tissues may reduce the degree of lipid autoxidation which occurs upon incubation. In this study, however, similar tissues from all groups within one experiment were analyzed at the same time.

The tissues were homogenized and incubated as described previously (BIERI and ANDERSON 1960). Briefly, 3 to 5 ml of a 5 % homogenate in phosphate buffer, pH 7.4, was pipetted into 50 ml Erlenmeyer flasks. These were incubated for one hour in a 37° C water bath, with shaking, in an atmosphere of air. Thereafter, 1 ml aliquots were pipetted

Table II. Summary of the incidences of vitamin E deficiency symptoms

Group no.	Basal diet no.	Source of protein	Addition to basal diet	Days on diet	Incidence[1] of		
					Exudative diathesis	Encephalo-malacia	White striation of breast muscles
2,304	2,304	Torula yeast, gelatine	None	22–32	9^{10}	1^{10}	
2,315			1.4 ppm SeO_2		0^{9}	0^{9}	
2,316			0.46 ppm SeO_2		0^{9}	1^{9}	
2,317			0.14 ppm SeO_2		0^{9}	1^{9}	
2,308			0.1 % Ethoxyquin		1^{10}	0^{10}	
2,311			0.01 % dl-α-Tocopheryl acetate		0^{9}	0^{9}	
2,343	2,343	Soybean-protein	None	21–33	15^{21}	0^{21}	16^{21}
2,344			0.3 % L-Cystine		8^{10}	0^{10}	0^{10}
2,345			0.1 % Ethoxyquin		0^{10}	0^{10}	0^{10}
2,346			0.46 ppm SeO_2		0^{10}	0^{10}	0^{10}
2,347			0.01 % dl-α-Tocopheryl acetate		0^{10}	0^{10}	0^{10}
2,383	2,383	Casein, gelatine	None	34–35	0^{10}	0^{10}	9^{10}
2,384			0.3 % L-Cystine		0^{10}	0^{10}	1^{10}
2,387			1.4 ppm SeO_2		0^{10}	0^{10}	3^{10}
2,389			0.01 % dl-α-Tocopheryl acetate		0^{10}	0^{10}	0^{10}

[1] The superscripts indicate the number of chicks in the group.

Table III. Thiobarbituric acid (TBA) values in homogenates of various tissues from chicks fed diets containing 60 % Torula yeast. Feeding period 22 to 32 days

Group	Addition to basal diet no. 2,304	No. of chicks	Mean TBA values ± standard error[1]				
			Liver[2]	Kidney[2]	Spleen[2]	Breast muscle[2]	Lungs[2]
2,304	None.....	7	356 ± 50	130 ± 29	225 ± 33	33 ± 13	24 ± 15
2,315	1.4 ppm SeO_2[4] ..	7	129 ± 14	117 ± 14	230 ± 12	36 ± 4	122 ± 9
2,316	0.46 ppm SeO_2 ..	7	111 ± 15	112 ± 6	226 ± 12	39 ± 4	155 ± 11
2,317	0.14 ppm SeO_2 ..	7	104 ± 16	129 ± 9	201 ± 10	45 ± 8	136 ± 8
2,308	0.1 % Ethoxyquin	7	19 ± 6	8 ± 1	31 ± 7	5 ± 1	11 ± 1
2,311	0.01 % dl-α-Tocopheryl acetate	7	20 ± 5	65 ± 14	22 ± 2	6 ± 2	13 ± 1

[1] Absorbancy at 530 mμ × 1,000 ± standard error.
[2] TBA test performed on the day of sacrifice.
[3] TBA values estimated on tissues stored 6 to 13 days at —20° C.
[4] 1.4 ppm SeO_2 = 1 ppm Se.

into 1.5 ml of 10 % trichloracetic acid. The extent of autoxidation of unsaturated fatty acids was estimated on the deproteinized solution as described previously using thiobarbituric acid (BIERI and ANDERSON 1960). The pink colors produced by the reaction of the reagent with malonic dialdehyde, an end-product of lipid peroxidation, were measured in a Beckman model C colorimeter. A reagent blank was carried through the color development procedure. The intensity of the color, and hence the amount of autoxidation, is reported as a thiobarbituric acid value (TBA value), which is the absorbancy reading at 530 mμ × 1,000.

Differences in TBA values between group means are considered significant only if the analysis of variance indicated a probability of less than 0.01.

Results

A high incidence of vitamin E-deficiency symptoms occurred in chicks fed each of the basal diets (cf. Table II). Thus, the Torula yeast and soybean protein diets produced a high incidence of exudative diathesis. The additions of selenium dioxide, ethoxyquin[1] or dl-α-tocopheryl acetate counteracted the exudates. The casein and soybean protein diets produced white muscle striations (DAM, PRANGE and SØNDERGAARD 1952). This latter symptom was counteracted by L-cystine, selenium dioxide, ethoxyquin and dl-α-tocopheryl acetate.

As reported previously (BIERI 1959, BIERI and ANDERSON 1960), the autoxidation in brain was higher than in any other tissue studied and was also completely unaffected by any dietary supplement. Consequently, the results with brain are not included and will not be considered when comparisons are made between other tissues.

The TBA values obtained from tissues of chicks fed three different levels of SeO_2, and also the antioxidant ethoxyquin, in the Torula yeast diet are shown in Table III. 0.1 % ethoxyquin was fully as effective as 0.01 % dl-α-tocopheryl acetate in preventing autoxidation in all tissues and may have been even more effective than vitamin E in the kidney. SeO_2 (0.14, 0.46 and 1.4 ppm), however, exerted an antioxidant action only in the liver but the effect was not as great as that of 0.01% dl-α-tocopheryl acetate or 0.1% ethoxyquin. The TBA values, which were similar for all three levels of SeO_2, represent a very pronounced decrease in autoxidation compared with the control, unsupplemented group.

An unexplained phenomenon was found in the lungs, where SeO_2 increased the autoxidation over that of the control group. This was not observed in succeeding experiments (below) with other diets.

In Table IV are shown the results obtained when L-cystine, ethoxyquin and SeO_2 were incorporated into the soybean protein diet. In this experiment (and also in the following one), in addition to incubating the homogenates alone an aliquot was also incubated with 0.1 μmole ascorbic acid per ml homogenate. Ascorbate has been shown to increase the autoxidation of fatty acids in tissues (OTTOLENGHI 1959). By incubating the homogenates both with

[1] 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline ("Santoquin"), Monsanto Chemical Co., St. Louis, Mo., U.S.A.

Table IV. Thiobarbituric acid (TBA) values in homogenates of tissues from chicks fed diets

Group no.	Addition to basal diet no. 2,343	Number of chicks	Mean values ± standard error[1]			
			Liver[2]		Kidney[2]	
			—C[4]	+C[4]	—C	+C
2,343	None	16	104 ± 14	140 ± 25	131 ± 8	238 ± 15
2,344	0.3 % L-Cystine	9	96 ± 29	112 ± 36	118 ± 11	231 ± 20
2,345	0.1 % Ethoxyquin	9	28 ± 3	32 ± 6	12 ± 2	13 ± 2
2,346	0.46 ppm SeO_2[5]	10	37 ± 6	37 ± 7	69 ± 10	123 ± 22
2,347	0.01 % dl-α-Tocopheryl acetate	10	22 ± 2	20 ± 2	37 ± 5	47 ± 6

[1] Absorbancy at 530 mμ × 1,000 ± standard error.
[2] Tissues were stored up to 74 days at —20° C before estimation of TBA values.
[3] TBA tests were performed on the day of sacrifice. Determinations with added ascorbic
[4] —C = no added ascorbic acid; +C = 0.1 μmole ascorbic acid added per ml homogenate.
[5] 0.46 ppm SeO_2 = 0.33 ppm Se.

and without ascorbic acid, it is possible to show that observed differences between the various groups are not due simply to differences in ascorbic acid content of the tissues (BIERI and ANDERSON 1960).

The most striking results in this experiment were those obtained with tissues from SeO_2-fed chicks. The antioxidant effect of SeO_2 on the liver was considerably more pronounced with the soybean protein diet than with the Torula yeast diet. In fact, the almost complete inhibition of autoxidation in the liver by SeO_2 was similar to that produced by ethoxyquin, and almost as complete as that produced by dl-α-tocopheryl acetate.

Table V. TBA values in homogenates of tissue from chicks fed diets containing 15 % casein and

Group no.	Addition to basal diet no. 2,383	Number of chicks	Mean TBA values ± standard error[1]			
			Liver		Kidney	
			—C[2]	+C[2]	—C	+C
2,383	None	10	41 ± 5	53 ± 6	58 ± 2	114 ± 8
2,384	0.3 % L-Cystine	8	47 ± 6	59 ± 9	58 ± 4	98 ± 9
2,387	1.4 ppm SeO_2[3]	10	52 ± 3	67 ± 3	57 ± 5	129 ± 12
2,389	0.01 % dl-α-Tocopheryl acetate	9	18 ± 1	20 ± 1	24 ± 3	30 ± 3

[1] Absorbancy at 530 mμ × 1,000 ± standard error
[2] —C = no added ascorbic acid; +C = 0.1 μmole ascorbic acid added per ml homogenate.
[3] 1.4 ppm SeO_2 = 1 ppm Se.
The tissues were stored up to 24 days at —20° C before estimation of TBA values.

containing 30 % soybean protein. Feeding period 21 to 33 days

Mean values ± standard error

Breast muscle[a]		Heart[a]		Lungs[a]		Spleen[a]
−C	+C	−C	+C	−C	+C	−C
44 ± 5	133 ± 8	78 ± 6	273 ± 12	37 ± 5	58 ± 7	149 ± 8
30 ± 6	73 ± 11	86 ± 9	287 ± 11	54 ± 8	71 ± 7	152 ± 10
36 ± 6	57 ± 10	19 ± 3	21 ± 3	29 ± 5	34 ± 6	89 ± 9
34 ± 6	68 ± 11	57 ± 5	216 ± 19	40 ± 5	48 ± 6	112 ± 15
32 ± 6	36 ± 7	14 ± 3	14 ± 2	13 ± 3	16 ± 3	11 ± 2

acid were not made.

A significant decrease in TBA values as a result of feeding SeO_2 was also noted in the kidney and heart both in the absence and presence of ascorbic acid.

In the third experiment, diet no. 2383 containing 15 % of casein, 10 % of gelatine and 1 % of lard (Table I), was used. This diet produced white muscle striation in 9 out of the 10 chicks (Table II). The TBA values of tissues from chicks fed this diet with cystine, SeO_2 or vitamin E are shown in Table V. Both cystine and SeO_2 produced a significant inhibition of lipid autoxidation in muscle. In addition, SeO_2 reduced the TBA value for heart but only in the absence of ascorbic acid.

10 % gelatine. Feeding period 34 to 35 days

Mean TBA values ± standard error

Breast muscle		Heart		Spleen	Lungs	
−C	+C	−C	+C	−C	−C	+C
37 ± 3	106 ± 8	65 ± 5	225 ± 10	101 ± 12	44 ± 3	61 ± 4
19 ± 1	68 ± 5	52 ± 3	214 ± 10	65 ± 11	38 ± 3	55 ± 3
25 ± 2	80 ± 5	45 ± 5	244 ± 12	129 ± 10	40 ± 2	67 ± 4
7 ± 1	7 ± 1	12 ± 1	14 ± 2	31 ± 3	11 ± 1	14 ± 1

Discussion

These results confirm and extend the previous observations (BIERI 1959) of the antioxidant effect of dietary selenite and L-cystine on certain tissues. In addition to the effect on the liver found before, it is apparent that under appropriate dietary conditions, as with the soybean protein diet, SeO_2 also inhibits autoxidation in other tissues, specifically, the heart and kidney. This action of SeO_2, however, is strongest in the liver but the results suggest that perhaps the action is widespread in the body. The observation in the experiment with Torula yeast that three different dietary levels of SeO_2 all inhibited autoxidation in the liver to the same degree suggests that the tissue concentration of biologically active selenium is not increased by higher supplements of the element.

It should be pointed out that the Torula yeast and soybean protein are deficient in biologically active selenium, since they can be used to produce exudative diathesis. The casein, however, contains sufficient selenium to prevent exudates with the level of lard used in this experiment.

The total selenium content of the various proteins determined by neutron activation analysis was for Torula yeast 0.038 μg, for casein 0.382 μg, for soybean protein 0.14 μg, and for gelatine 0.088 μg selenium per gram substance (SCHWARZ 1960).

Further, it should be emphasized that the Torula yeast contains 2.4 % dienoic and 0.14 % trienoic fatty acids (DAM et al. 1957), the soybean protein contains 0.65 % dienoic and 0.07 % trienoic whereas casein is practically devoid of polyenoic fatty acids.

In the case of L-cystine, its antioxidant effect seems restricted to the muscle. The restriction of the effect to muscle is in accord with the nutritional role of L-cystine in chicks in that the only vitamin E-deficiency symptom it will prevent is white muscle striation.

The effectiveness of the antioxidant ethoxyquin in inhibiting autoxidation in all tissues (except brain) agrees with the observation that this compound at least in some respects will replace vitamin E in the chick (MACHLIN et al. 1959, SØNDERGAARD et al. 1960).

Although we do not know the precise mechanism whereby dietary SeO_2 and L-cystine exert their antioxidant action in tissues, there is sufficient evidence to permit some hypotheses to be made. Since it is known (BIERI 1959) that selenite and L-cystine added to tissue homogenates will not inhibit lipid autoxidation, it is apparent that these substances when fed either alter the normal chemical composition of the cell or give rise to new or modified compounds which act as antioxidants. Since cystine and selenium are incorporated into cell proteins, the latter postulate seems more feasible although the two probably have different sites of action. In the case of selenium, it may be that the substitution of selenium for sulfur in the proteins, particularly in the liver, yields a new protein with remarkable antioxidant properties.

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Effect of Reserpine on the Storage of New-formed Catecholamines in the Adrenal Medulla

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Abstract

BERTLER, Å., N.-Å. HILLARP and E. ROSENGREN. *Effect of reserpine on the storage of new-formed catecholamines in the adrenal medulla.* Acta physiol. scand. 1961. *52.* 44—48. — When L-dopa (100 mg/kg body wt.) was given i.v. to reserpinized (5 mg/kg) rabbits, appreciable amounts of dopamine and noradrenaline rapidly formed in the adrenal medulla. The results support the view that reserpine does not — at least not to a great extent — interfere directly with the decarboxylating and β-hydroxylating steps in the amine synthesis. To a great extent, however, the drug prevented the new-formed amines from being incorporated in the storage granules, an incorporation which in normal animals is a very rapid and efficient process. It seems reasonable to assume that reserpine in some way blocks the process of monoamine storage. This may well be the main site of action of the drug.

Reserpine causes a general depletion of the monoamine stores in the body. Mainly on the basis of experiments with blood platelets it has been suggested that the primary effect of this drug on 5-hydroxytryptamine (5-HT) is on the storage mechanism (*cf.* SHORE *et al.* 1957). It has, however, hitherto been difficult to test this theory by experiments *in vivo*.

A new method of studying the amine storage problem has recently become available when it was shown that large amounts of noradrenaline (NA) and dopamine (DA) are rapidly formed and stored in the adrenaline (A) cells of the rabbit suprarenal medulla after an intravenous injection of L-3,4-dihydroxyphenylalanine (dopa) (BERTLER, ROSENGREN and ROSENGREN 1960, BERTLER, HILLARP and ROSENGREN 1960 a, b). In the present paper it is shown that reserpine interferes with the storage of amines formed in this way.

Material and methods

An i.v. injection of reserpine ("Serpasil", Ciba, 5 mg/kg body wt.) was given to rabbits (1.5 to 2 kg body wt.). After various periods of time L-dopa (100 mg/kg) was injected i.v. and the animals were killed 30 min to 2 hrs later by an i.v. injection of air. The adrenal medullas from each animal were homogenized in 0.3 M sucrose and centrifuged as described previously (BERTLER, HILLARP and ROSENGREN 1960 a). Catecholamines were determined spectrophoto-fluorimetrically (BERTLER, CARLSSON and ROSENGREN 1958, CARLSSON and WALDECK 1958.)

In 4 animals the spinal cord was transected at C6 (Nembutal narcosis) immediately before the administration of dopa.

Results and discussion

When dopa was administered to rabbits 3 to 48 hours after an i.v. injection of reserpine, appreciable amounts of NA and DA rapidly formed in the adrenal medulla (Table I). The amounts found, however, are generally lower than those found in normal animals injected with dopa (see BERTLER, HILLARP and ROSENGREN 1960 a). This could mean that reserpine in some way interferes with the amine synthesis. It has been shown, however, that denervation of the rabbit adrenal medulla largely prevents the amine depletion caused by reserpine (KRONEBERG and SCHÜMANN 1957). It is thus probable that the secretory nerves of the medulla are continuously operating in a reserpinized animal. Consequently, unknown amounts of amines formed after a dopa injection may be lost by secretion. This is in good agreement with the finding that larger amounts of NA were present in the two animals in which a transection of the spinal cord at C6 was made immediately before the dopa injection (Table I).

Thus the results support the view that reserpine does not — at least not to a great extent — interfere directly with the decarboxylating and β-hydroxylating steps in the amine synthesis. This is not unexpected since it has been shown that 5-HT forms in reserpinized animals in an apparently normal way (SHORE, SILVER and BRODIE 1955). There is a possibility, however, that reserpine may indirectly disturb the formation of NA and A. It has been suggested that the β-hydroxylation of DA occurs in the storage granules (KIRSHNER 1959). If this is true, reserpine may well depress this reaction to some extent since it inhibits the uptake of DA in the granules (see below).

The amines formed from injected dopa in normal rabbits are taken up and stored very rapidly by the storage granules. In reserpinized animals, on the contrary, the new-formed DA and NA were mainly recovered "free" in the "cytoplasmic sap" (Table I). Thus to a large extent the drug prevents the amines from being incorporated in the storage granules. This inhibition of the storage process seems to be far from complete even 24 hours after the injection of reserpine since most of the NA is particle-bound 2 hours after the dopa administration. The values actually found are probably misleading,

Table I. Content (μg/kg body wt) and intracellular distribution of catecholamines in the adrenal The mean value is given together with the lowest and highest value observed in each group (within In 4 animals the spinal cord was transected at C6 immediately before the dopa administration

Number of Animals	Treatment of Animals	Adrenaline	
		Total μg	"Free" Per cent
2	Res. 3 hr, Dopa 30 min	43 (41–46)	15 (12–17)
2	Res. 5 hr, Dopa 30 min	19 (15–23)	21 (18–24)
4	Res. 20 hr, Dopa 30 min	3.2 (1.8–6.4)	34 (20–57)
3	Res. 48 hr, Dopa 30 min	4.7 (2.0–9.3)	22 (13–31)
2	Res. 3 hr, Dopa 1 hr	37 (32–42)	15 (14–17)
2	Res. 5 hr, Dopa 1 hr	27 (25–29)	20 (18–22)
6	Res. 20 hr, Dopa 1 hr	4.1 (2.4–5.6)	23 (18–28)
2	Res. 20 hr, C6 trans., Dopa 1 hr	2.6 (2.0–3.2)	62 (51–72)
2	No res., C6 trans., Dopa 1 hr	38 (29–48)	8 (11– 6)
4	Res. 24 hr, Dopa 2 hr	6.3 (3.0–9.1)	24 (14–49)
4	Res. 25 hr	2.6 (0.6–6.0)	12 (6–14)
1	Res. 72 hr	3.9	18
1	Res. 96 hr	9.2	6
1	Res. 120 hr	17	14
1	Res. 144 hr	23	11

however, since unknown amounts of the new-formed amines may have been secreted, as pointed out above. The result will be that after some time the fraction of "free" amines has largely disappeared. The fact that the largest amounts of "free" amines accumulated in the animals with spinal cord transection clearly supports this view. Furthermore, these amines may also be destroyed enzymatically, *e. g.* by monoamine oxidase, which may be present in the medullary cell.

It may well be, however, that reserpine — not even with the high dosage used — is capable of more than a fairly incomplete prevention of the storage of new-formed amines in the adrenal medulla. This effect may be much more pronounced in other catecholamine-producing tissues since the medullary cells are particularly insensitive to reserpine (CARLSSON *et al.* 1957).

The results are in perfect agreement with those obtained by BRODIE *et al.*

medulla of reserpinized (5 mg/kg) rabbits after administration of L-dopa (100 mg/kg). parentheses).

Noradrenaline		Dopamine	
Total μg	"Free" Per cent	Total μg	"Free" Per cent
0.7 (0.5–0.9)	100	1.7 (1.4–1.9)	43 (42–44)
2.0 (1.2–2.8)	60 (54–65)	2.0 (1.4–2.5)	53 (49–57)
1.8 (1.3–2.4)	62 (53–78)	3.2 (1.8–3.8)	78 (73–82)
4.3 (3.0–5.3)	40 (21–50)	1.3 (0.8–1.6)	45 (21–60)
2.8 (1.4–4.2)	22 (14–30)	1.0 (0.7–1.3)	31 (29–33)
1.9 (1.6–2.2)	28 (21–35)	0.7 (0.6–0.8)	26 (25–27)
2.0 (0.3–3.7)	74 (63–100)	1.8 (0.3–4.5)	60 (43–69)
4.8 (3.6–6.0)	67 (65–69)	1.4 (1.2–1.7)	56 (50–62)
5.5 (3.5–7.5)	13 (12–15)	2.5 (1.8–3.2)	5 (3–7)
1.9 (1.1–2.7)	37 (25–71)	1.3 (0.2–2.4)	67 (20–92)
0.1 (0.0–0.3)	10 (0–20)	0.15 (0.1–0.2)	50 (50–50)
1.3	25		
1.7	13		
3.5	21		
0.9	13		

(1957) and HUGHES, SHORE and BRODIE (1958), who showed that reserpine prevents the uptake of 5-HT by blood platelets *in vitro*. Thus it seems reasonable to assume that reserpine in some way blocks the process of monoamine storage. This may well be the main site of action of the drug. The fact that reserpine behaves like a liberating agent, causing a depletion of the stores, does not contradict such a view. The two processes: storage and release, are presumably balanced in the normal cell. Thus if the storage process is blocked the result will be a depletion, the rate of which must be dependent *i. a.* on the secretory activity of the cell. In the brain, the monoamines have a high turnover (UDENFRIEND and WEISSBACH 1958, CARLSSON 1960) and consequently a blocking of the storage process will rapidly cause their disappearance.

The effect of reserpine on isolated amine granules will be discussed in another paper.

The content and intracellular distribution of amines in the medulla of rabbits, to which only reserpine had been given, were also studied (Table I). Very small amounts of NA and DA were present 25 hours after the injection, and stored amines appeared during the following days much more slowly than after an insulin depletion (see BERTLER, HILLARP and ROSENGREN 1960 a). The slow recovery, however, may not entirely be a result of an inhibition of the storage process since this inhibition seems to be rather weak 48 hours after the administration of reserpine as judged by the experiments with dopa injection.

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Phenylalanine and Tyrosine in the Adrenal Medulla

By

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Abstract

HALL, G., N.-Å. HILLARP and G. THIEME. *Phenylalanine and tyrosine in the adrenal medulla.* Acta physiol. scand. 1961. *52.* 49—52. — The claim (FELLMAN 1958, FELLMAN and DEVLIN 1958) that large amounts of free phenylalanine are present in the adrenal medulla has been tested using cation exchange and paper chromatographic methods. The concentration of this amino acid was found to be very low (< 20 to 40 μg/g wet wt). Tyrosine — but no m-tyrosine, phenylserine or 3,4-dihydroxyphenylalanine (< 20 μg/g) — was detected in small amounts (10 to 20 μg/g). Thus the catecholamine producing cells in the gland do not accumulate free phenylalanine or tyrosine to a level appreciably higher than that in the blood plasma.

FELLMAN and DEVLIN (1958) and FELLMAN (1958) have reported the presence of large amounts of free phenylalanine in the adrenal medulla. This finding — if correct — would suggest that the catecholamine synthesis in the gland starts from phenylalanine and not from tyrosine as generally believed.

By use of more suitable chromatographic methods than those applied by FELLMAN it is shown in this paper that the concentration of both phenylalanine and tyrosine in the adrenal medulla does not materially exceed that in the blood plasma.

Material and Methods

Extraction procedure. Pooled adrenal medullas of cow were thoroughly extracted with perchloric acid (PCA; final concentration 0.4 N). After centrifugation the extract was neutralized at 0° to about pH 3—4 with potassium carbonate. In some experiments extraction was performed with 0.1 N HCl according to the procedure of UDENFRIEND and COOPER (1953).

Ion exchange chromatography. Two different procedures were used.

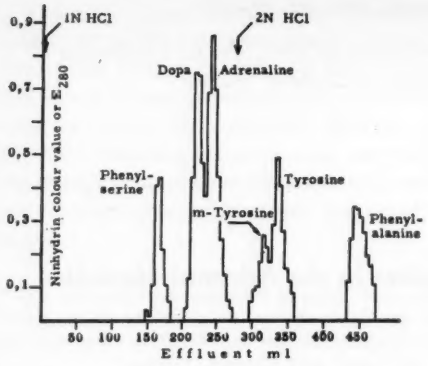

Fig. 1. Cation exchange chromatography (Dowex 50 W—X 8, 100 × 0.6 cm, H^+) of phenylserine, m-tyrosine, tyrosine, phenylalanine (0.5 mg of each), dopa and adrenaline (1 mg of each).

Method I. To the extract from 1 g (wet wt) of medulla, acetic acid was added to a final concentration of 1 N. The solution was transferred to a cation exchange column (Dowex 50W—X8, 200—400 mesh, 30 × 0.6 cm, H^+) with a small portion of N acetic acid. The amino acids were eluted (4.6 ml fractions) with 1 M acetic acid — sodium acetate buffer of pH 4.0. Ninhydrin positive substances — determined according to the method of MOORE and STEIN (1954) — were eluted in three sharp peaks. Phenylalanine and tyrosine (added to the extract) appeared in the last and biggest peak when 100 to 110 ml of the buffer had passed through the column.

Method II. The extract was acidified with HCl (final concentration 1 N) and applied to a longer column (100 × 0.6 cm). The elution was performed first with 1 N HCl until the catecholamines (determined by ultraviolet absorption measurements) were completely eluted and then with 2 N HCl. Practically the whole amount of the ninhydrin positive components were recovered in various fractions of the N acid. Meta-tyrosine, tyrosine and phenylalanine (100 to 500 μg of each added to the extract) appeared in fairly sharp peaks and free from substances interfering in the paper chromatographies when about 20, 50 and 150 ml of the second eluant had passed through the column. When added to the extract, phenylserine was eluted with the N acid in a sharp peak before 3,4-dihydroxyphenylalanine which emerged — together with noradrenaline — immediately before the adrenaline peak. — A chromatogram of a synthetic mixture of amino acids is shown in Fig. 1.

In two experiments an extract from 5 g of medulla was chromatographed according to method I on a larger column (40 × 1.3 cm). All the fractions with ninhydrin positive substances of the third peak were pooled and evaporated to dryness *in vacuo* at + 40° after acidification with HCl. The residue was thoroughly extracted with 95 % ethanol containing 1 % HCl. After evaporation of most of the ethanol with N_2, the material was dissolved in N HCl and re-chromatographed according to method II after addition of 500 μg of adrenaline, used as a guide for the development of the chromatogram.

When HCl was used as eluant, aliquots of the fractions were evaporated to dryness *in vacuo* over sodium hydroxide before the ninhydrin determinations were made.

Paper chromatography. The fractions containing the various peaks of ninhydrin positive substances were evaporated to dryness *in vacuo* at + 40° by use of a rotating evaporator. When the acetate buffer was used as eluant the fractions were first acidified with an appropriate amount of HCl. The residue was taken up in a small volume of

acid ethanol or 0.1 N HCl and applied to Whatman no. 1 paper (washed with HCl and sodium ethylenediaminetetraacetate). The material was subjected to ascending chromatography in two systems: n-butanol — glacial acetic acid — water 4 : 1 : 5 and isopropanol — aqeous ammonia (25 % NH_3) — water 80 : 2 : 18. The spots were located by spraying with a 0.2 % solution of ninhydrin in ethanol or — for location of phenolic compounds — with diazotized p-nitroaniline according to STUDNITZ and HANSON (1959). Pure amino acids (2—10 μg) were used as references.

Determination of tyrosine. After ion exchange chromatography tyrosine was determined colorimetrically in the eluted fractions using 1-nitroso-2-naphtol according to UDENFRIEND and COOPER (1952).

Recovery experiments. Only small losses, or none at all, were found on ion exchange chromatography of pure amino acids as shown by direct determinations with ninhydrin. In four experiments, tyrosine (100 μg) and phenylalanine (100—500 μg) were added to 1 g of gland tissue which was then extracted with PCA. Ion exchange (method II) and paper chromatography were performed as described above. The recovery was at least 70 %.

Results and Discussion

FELLMAN and DEVLIN (1958) found very large amounts of free phenylalanine in the beef adrenal medulla (900 to 1,000 μg/g wet wt). The chemical determination used, however, is unspecific and the present authors have found the identification of the amino acid by direct paper chromatography of an unfractionated tissue extract to be unreliable. Two cation exchange procedures were therefore devised in order to remove interfering substances. In one of them (method II) the catecholamines and essentially the whole amount of ninhydrin positive compounds and other interfering components could be removed before tyrosine and phenylalanine — added to the extract — were eluted in two well separated and fairly sharp peaks (see Fig. 1). In spite of the fact that phenylalanine added to the gland tissue before the extraction took place was easily detected and recovered in good yield, no phenylalanine was found in the adrenal medulla. Since the methods used admitted of detection of 20 to 40 μg in an extract from 1 g of medulla, the concentration of this amino acid in the gland must be very low. In fact, it is probably as low as that in other tissues and blood plasma (see STEIN and MOORE 1954, TALLAN, MOORE and STEIN 1954).

Tyrosine — but no m-tyrosine, phenylserine or 3,4-dihydroxyphenylalanine ($< 20\,\mu$g/g) — was detected in small amounts. Its concentration in the gland was estimated to be in the order of 10 to 20 μg/g, *i. e.* about the same as that in other tissues and blood plasma.

Thus the cells in the adrenal medulla do not accumulate free phenylalanine or tyrosine to any appreciable extent. This may mean that the amino acid used as starting material for the catecholamine production is drawn directly from the blood when it is needed. It has been shown (ROSENFELD, LEEPER and UDENFRIEND 1958) that the cells can take up tyrosine from the blood and utilize it in the amine synthesis. There is thus at present no reason to believe

that this synthesis starts from phenylalanine. The fact that slices of adrenal medulla are able to convert phenylalanine to tyrosine does not contradict this view since this conversion was found to be due to a non-enzymatic and presumably non-specific reaction (FELLMAN and DEVLIN 1958).

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The Functional State of Vasomotor Nerves to Skeletal Muscle Vessels in Reserpinized Cats

By

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Recieved 20 January 1961

Abstract

ROSELL, S. and A. ROSÉN. *The functional state of vasomotor nerves to skeletal muscle vessels in reserpinized cats.* Acta physiol. scand. 1961. *52.* 53—61. — The functional state of vasomotor nerves to skeletal muscle vessels was studied in anesthetized cats treated with reserpine. The effects of the vasoconstrictor nerves could be completely blocked by reserpine while the responses of the vasodilator nerves seemed to be unaffected. The results are consonant with the view that there are two types of vasomotor nerves to the skeletal muscle vessels; namely, adrenergic vasoconstrictor nerves and cholinergic vasodilator nerves. The cardiovascular changes evoked by reserpine are discussed.

According to numerous investigators, there are specific vasodilator fibers in the sympathetic nerves to the skeletal muscle vessels of the cat. In view of the postganglionic nerve terminals, BÜLBRING and BURN (1935, 1936) and ROSENBLUETH and CANNON (1935) claim that both adrenergic and cholinergic vasodilator nerves exist in the cat, while FOLKOW and UVNÄS (1950) argue that the skeletal muscle vessels have only cholinergic vasodilator nerves. On the other hand, P. CANNON, RAULE and SCHAEFER (1954) still question the existence of vasodilator nerves. They adhere to the view that vasodilatation is due to inhibition of vasoconstrictor nerve activity.

Reserpine has been found to deplete various organs of dopamine, adrenaline, noradrenaline and 5-hydroxytryptamine (CARLSSON *et al.* 1958, BERTLER, CARLSSON and ROSENGREN 1956, HOLZBAUER and VOGT 1956, SHORE, SILVER

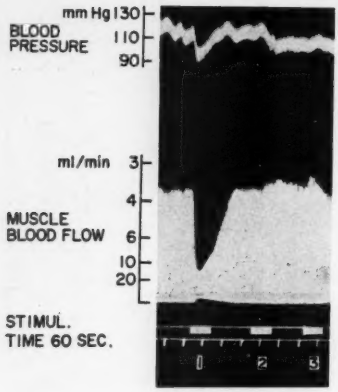

Fig. 1. Cat 3.5 kg. Chloralose-Urethane.
Effect on muscle blood flow of stimulation of the sympathetic chain after reserpine treatment (0.05 mg/kg/day for 5 days).
1. Stimulation, 2.5 V, 5 imp/sec.
2. Atropine, 0.5 mg/kg I. V.
3. Stimulation, 2.5 V, 5 imp/sec.
Note vasodilatation blocked by atropine.

and BRODIE 1955). BERTLER *et al.* (1956) and MUSCHOLL and VOGT (1958) have demonstrated that following reserpine treatment the adrenergic neurones lose their transmitter substance. This action of reserpine has been used in our study designed to reinvestigate whether specific vasodilator nerves exist and if so, what type of postganglionic fibers they possess. Our experiments also yielded information on certain cardiovascular actions of reserpine, which will be discussed in the following.

Methods

Reserpine Treatment

From one to six days prior to the experiments, reserpine (Serpedin®, Pharmacia) was administered subcutaneously in daily doses of from 0.025 to 0.5 mg/kg. In the animals which received only a single injection, 2—5 mg/kg was given s. c. 20—24 hours before the experiment.

Preparation

Cats weighing between 2.0 and 4.5 kg were anesthetized with urethane *i.v.* (0.4—1 g/kg b. w.) In animals used for studying vasomotor reflexes, chloralose (50 mg/kg) supplemented by 20 per cent urethane (usually 0.4—0.8 g) was administered. The trachea was cannulated. Arterial pressure was recorded by a mercury manometer or a pressure transducer (Statham P 23 AA) connected to the carotid artery. Heart rate was determined with an interval recorder via impulses from the blood pressure channel of a Grass polygraph (GOLDSCHMIDT and LINDGREN 1961). Rectal temperature was maintained at 36—37° C by radiant heat from a heating lamp. Blood flow to the musculature was recorded in the femoral artery of a skinned limb, with the aid of a silicone-filled drop counter operating an ordinate recorder (LINDGREN 1958). To maintain warmth and moistness, the skin was replaced around the muscle. A ligature around the ankle isolated the paw from the circulation. In some experiments a cross-circulation technique was used. A femoral artery in the donor animal was connected via a drop counter to the

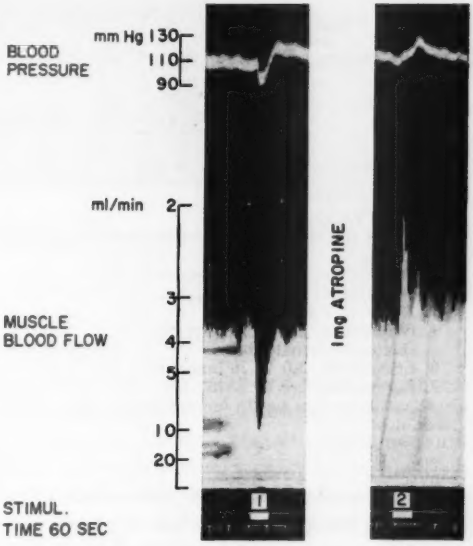

Fig. 2. Cat 2.5 kg, Chloralose-Urethane.
Effect on muscle blood flow of stimulation of the sympathetic chain after reserpine treatment (0.05 mg/kg/day for 4 days).
1. Stimulation, 2.0 V, 20 imp/sec.
2. Stimulation, 2.0 V, 20 imp/sec.
Note vasodilatation, after atropine, vasocontriction.

corresponding femoral artery in a skinned hind limb of the recipient animal, and the blood flow to the muscles thus recorded. The cross-circulated leg was drained by a plastic tube from the femoral vein of the recipient to the corresponding vein of the donor animal. To prevent clotting, heparin (25 mg/kg) was given intravenously. All values were recorded on a Grass polygraph or on a kymograph.

The sympathetic chain, isolated and cut via the anterior approach, was stimulated in the distal part with a bipolar silver electrode at the level of L_4—L_5. The carotid sinus nerve or the central part of a transected vagus nerve was isolated and stimulated with a bipolar silver electrode. For hypothalamic stimulation of the sympathetic vasodilator pathway a unipolar stainless steel electrode, oriented by means of the Horsley-Clarke technique, was used. Electrical stimulation was produced by a square-wave generator of 1,000 ohms output resistance. The duration of impulses, the voltage and the frequency were independently variable. Dextran (Macrodex®, Pharmacia) was administered intravenously as required, to compensate for blood loss. Artificial respiration was given during reflex activations.

Results

The results are based on data from 51 experiments. In 34 of these the sympathetic chain was stimulated. Vasomotor reflexes were produced in 14 cats, and in 3 the sympathetic vasodilator pathway in the hypothalamus was activated.

1. Sympathetic Chain Stimulation

In cats, stimulation of the distal portion of the cut sympathetic chain at the level of $L_4 - L_5$ usually causes contriction of the skeletal muscle blood vessels

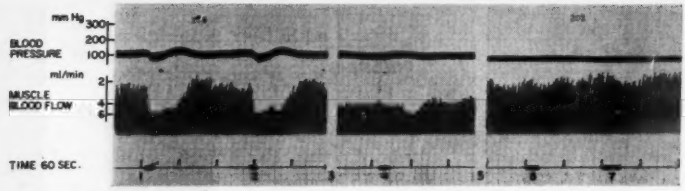

Fig. 3. Cat 3.0 kg. Urethane.
Effect on muscle blood flow of stimulation of the hypothalamic vasodilator pathway in reserpinized cat (5 mg/kg).
1. Stimulation, 2.0 V, 70 imp/sec.
2. Stimulation, 2.0 V, 70 imp/sec.
3. Atropine, 0.4 mg/kg I. V.
4. Stimulation, 2.0 V, 70 imp/sec.
5. Bilateral adrenalectomy.
6. Stimulation, 2.0 V, 70 imp/sec.
7. Stimulation, 2.0 V, 70 imp/sec.
Note vasodilatation partly blocked by atropine; after bilateral adrenalectomy no changes in blood flow.

of the hind limb. In reserpine-treated cats, however, the same stimulation produced vasodilatation when the reserpine dose was adequate. Fig. 1 illustrates an experiment in which the animal received daily doses of reserpine 0.05 mg/kg for 5 days. Sympathetic chain stimulation caused pronounced vasodilatation which was blocked by atropine — a finding which suggests the existence of cholinergic vasodilator nerves. The fact that sympathetic chain stimulation caused a blood flow increase does not preclude the possibility that the transmission mechanism at the adrenergic vasoconstrictor nerve endings was still functioning, since the effect of vasoconstrictor nerve stimulation may be concealed by the increased blood flow. That this was not the case, however, is evident from the fact that stimulation following atropine did not reduce the blood flow. An example of vasoconstriction with repeated stimulation after atropine, is illustrated in Fig. 2. In this experiment the vasodilator effect predominated even though the effect of vasoconstrictor nerve stimulation remained, the dose of reserpine (0.05 mg/kg/day for 4 days) apparently having been insufficient to abolish it completely. Following high doses of reserpine (2—5 mg) the effects of vasoconstrictor nerve stimulation were always abolished.

2. Hypothalamic Stimulation

The sympathetic vasodilator system has relay stations between the cerebral cortex and the peripheral outflow. Synapses are thought to exist in the hypothalamus and in the mesencephalon (Eliasson, Lindgren and Uvnäs 1954, Lindgren 1955). In order to ascertain whether the hypothalamic-spinal part of the vasodilator system is functionally intact or blocked at some point in reserpine-treated animals, the vasodilator pathway was stimulated in the anterior hypothalamus of reserpinized cats (5 mg/kg). Stimulation still evoked vaso-

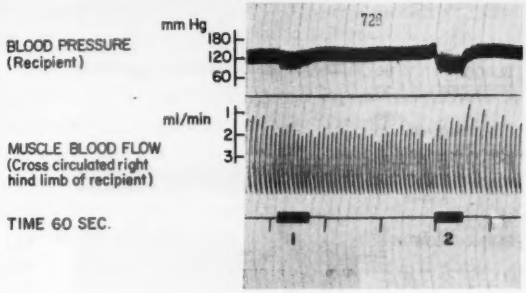

Fig. 4. Cat 3.7 kg. Chloralose-Urethane.
Effect on blood pressure and muscle blood flow of stimulation of the carotid sinus nerve in reserpinized cat (5 mg/kg).
1. Stimulation, 3 V, 10 imp/sec.
2. Stimulation, 3 V, 15 imp/sec.
Note no change in muscle blood flow; decrease in blood pressure.

dilatation which was blocked by atropine, thus indicating activation of cholinergic vasodilator nerves (Fig. 3). Although stimulation following atropine still increased the blood flow, the latency was now longer, indicating that a humoral factor was involved. After bilateral adrenalectomy, stimulation elicited no change in blood flow. It seems, therefore, that even when the animal had received a large dose of reserpine (5 mg/kg) catechol amines might have been released from the adrenals by hypothalamic stimulation. This is consistent with the finding of MUSCHOLL and VOGT (1958) that even high doses of reserpine do not entirely deplete the cat's adrenal medulla of its catechol amines.

3. Reflex Activation

After having demonstrated that the vasodilator pathway was intact in reserpinized cats from the hypothalamus to the peripheral termination but that the adrenergic vasoconstrictor effects were blocked, we initiated a series of experimental attempts to increase muscle blood flow reflexly. In the event of such increase, vasodilatation would be attributable to activation of specific cholinergic vasodilator nerves rather than to inhibition of vasoconstrictor tone.

Reflex activation was produced by afferent stimulation of one of the carotid sinus nerves or one of the vagi. In order to prevent a blood-pressure fall from influencing the blood flow in the peripheral area, a cross-circulation technique was utilized. In the experiment shown in Fig. 4 the skinned right hind limb was cross circulated. The recipient animal in this case had received reserpine (5 mg/kg) the day before the experiment. The fact that stimulation of the carotid sinus nerve did not alter the blood flow means that the sympathetic vasodilator nerves were not activated. On the other hand, there was a blood-pressure fall in the recipient cat, indicating either bradycardia or a reduction of peripheral resistance in vascular areas other than skeletal muscle. — Studies on skin and intestinal blood flow indicate that in those areas as well, no increase of blood flow can be induced by afferent stimulation of the vagus or carotid sinus nerve after reserpine treatment (ROSELL and ROSÉN, unpublished observa-

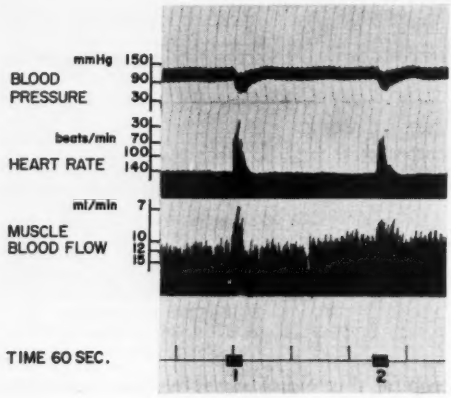

Fig. 5. Cat 4.2 kg. Chloralose-Urethane. Effect on heart rate and muscle blood flow of stimulation of the carotid sinus nerve in reserpinized cat (5 mg/kg).
1. Stimulation, 2.5 V, 15 imp/sec.
2. Stimulation, 2.5 V, 10 imp/sec.
Note reflex bradycardia.

tions). — However, in reserpinized cats carotid sinus nerve stimulation induced bradycardia (Fig. 5). Thus the blood-pressure fall seen in Fig. 4 presumably results from a reflexly induced bradycardia.

Discussion

Sympathetic chain stimulation in reserpine-treated cats produces an increased blood flow in skeletal muscle vessels (Fig. 1), in marked contrast to the vasoconstrictor response usually seen in untreated cats. The disappearance of the vasoconstrictor response is presumably due to loss of the transmitter substance from the adrenergic neurones following reserpine treatment (BERTLER *et al.* 1956, MUSCHOLL and VOGT 1958). If this loss is of sufficient magnitude, adrenergic nerve impulses will be incapable of producing responses in the effector organ. Thus, if cholinergic vasodilator nerves exist, their effects should predominate when the sympathetic chain is stimulated. Even relatively small doses of reserpine (0.05 mg/kg/day for 4 days) are sufficient to cause predominance of the effects of the vasodilator nerves (Fig. 2). When the cholinergic vasodilator nerve response has been blocked by atropine, renewed sympathetic stimulation provides information about the functional state of the adrenergic constrictor nerves (Fig. 1 and 2).

It seems evident that reserpine affects the adrenergic vasomotor neurones peripherally. Investigations by others authors, using different methods, lead to similar conclusions. BERTLER *et al.* (1956) showed that stimulation of the sympathetic ganglia by carbacholine failed to increase the blood pressure of atropinized cats treated with reserpine. This may indicate that the transmission mechanism at the adrenergic nerve endings was no longer intact, due possibly to the depletion, by reserpine, of the transmitter substance in the

adrenergic nerves. MUSCHOLL and VOGT (1958) have shown, moreover, that peripheral adrenergic neurones lose their noradrenaline after injection of reserpine.

Fig. 1 shows that sympathetic chain stimulation induces vasodilatation in cats treated with reserpine and that the vasodilatation is blocked by atropine. These facts strongly indicate the existence of specific cholinergic vasodilator nerves to the skeletal muscle vessels. This is contradictory to the assumption that in the cat most of the sympathetic vasodilator fibers to the skeletal muscles are adrenergic (BÜLBRING and BURN 1935, 1936, ROSENBLUETH and CANNON 1935), but supports the view of FOLKOW and UVNÄS (1950) that the sympathetic vasodilator nerves are cholinergic.

P. CANNON *et al.* (1954) have questioned the existence of specific vasodilator nerves and have argued that the vasodilator effects during stimulation of the sympathetic system stem from inhibition of vasoconstrictor nerve activity. This explanation appears to be invalidated by our experiments. The cross-perfusion experiments in which afferent stimulation of the carotid sinus nerve or vagi was applied, show that when the dose of reserpine is sufficient there is no vasoconstrictor nerve activity which can be inhibited. It is still possible, however, to induce vasodilatation of skeletal muscle vessels by sympathetic chain stimulation or by appropriate stimulation in the hypothalamus.

Thus these experiments support the view of LINDGREN and UVNÄS (1955) that skeletal muscle vasodilatation may be elicited via sympathetic nerves in two different ways; namely, by inhibition of the adrenergic vasoconstrictor nerve activity and by activation of cholinergic vasodilator nerves. They also lend weight to the theory that sympathetic vasodilator nerves are not involved in depressor reflexes elicited from the carotid sinus region or from the aortic arch (Fig. 4).

It is interesting to note in cats treated with high doses of reserpine (5 mg/kg) that the vasodilator pathway is intact from the hypothalamus to the peripheral outflow, despite the fact that the central nervous system is presumably depleted of dopamine, noradrenaline, adrenaline and 5-hydroxytryptamine. It has been suggested that some of these biogenous substances might function as chemical mediators within the central nervous system (BRODIE and SHORE 1956). Within the peripheral nervous system reserpine blocks the transmission at the adrenergic nerve endings by releasing the transmitter substance. By analogy, one might assume that the transmission would also be blocked at the synapses in the central nervous system, where any one of the substancess released by reserpine might act as a chemical mediator. It is doubtful, therefore, whether any of the afore mentioned substances functions as a chemical mediator within the sympathetic vasodilator system.

The fact that afferent stimulation of the vagus or of a carotid sinus nerve produces no reflex vasodilatation in reserpinized cats could be explained by SCHNEIDER's (1955) hypothesis that reserpine blocks afferent stimuli, preventing

them from reaching sympathetic centers, or by BEIN's (1953 and 1955) suggestion that reserpine activates central inhibitory sympathetic structures. Both authors found that after administration of reserpine the blood-pressure response to activation of the carotid occlusion reflex was diminished. HORWITZ, KUSKIN and WANG (1959) recently reported that pressor responses from medullary and hypothalamic vasomotor areas were reduced in reserpine-treated cats. They postulate a general depressive effect of reserpine on the central vasomotor mechanism. ANAND, DUA and MALHOTRA (1957) and HARRISON and GOTH (1956) hold, in general, a similar view concerning the action of reserpine. The results of these experiments might well be explained by partial or total inactivation of peripheral adrenergic efferent neurones. Since the carotid occlusion reflex not only affects the vasomotor tone of peripheral vessels but also changes the heart rate, it is difficult to analyse the underlying mechanism of reserpine's circulatory effects by studying changes in blood pressure. To judge from our results, reserpine does not interfere with transmission in the synapses which relay impulses to the efferent part of the reflex arc studied, since stimulation of the carotid sinus nerve invariably induced pronounced bradycardia. That reserpine reduces the blood-pressure response during the carotid occlusion reflex, may be explained by the fact that the reflex increase in vasoconstrictor tone and in heart rate is diminished or precluded because the efferent adrenergic neurones are no longer intact.

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Acta physiol. scand. 1961. 52. 62—64

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Occurrence and Distribution of Catecholamines in the Fish Brain

By

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Abstract

Euler, U. S. v., *Occurrence and distribution of catecholamines in the fish brain.* Acta physiol. scand. 1961. *52.* 62—64. — Relatively high amounts of catecholamines were found in the brains of an elasmobranch, *Squalus acanthias* and a teleost, *Gadus callarias.* Only small quantities could be demonstrated in the brain of a cyclostome, *Myxine glutinosa.* Both adrenaline and noradrenaline occurred in the brains of *Squalus* and of *Gadus* although noradrenaline was predominant. A characteristic distribution of the catecholamines in the brain of *Squalus* was observed. The highest amount of noradrenaline was found in the diencephalon, hypothalamus, and hypophysis, while the cerebellum contained only very small quantities.

Noradrenaline and small amounts of adrenaline have been demonstrated in the brain of mammals (*cf.* Euler 1956). Vogt showed in 1954 that certain regions of the brain like the hypothalamus contained larger amounts of amines than others. It is still uncertain whether all of the noradrenaline in the brain is present in postsynaptic neurons, *e. g.* vasomotor fibres, or whether part of it occurs in chromaffin cells or in other stores. As to the adrenaline it may be postulated that it is present in chromaffin cells, since there is no evidence for its occurrence in axons.

The present study was made in order to find out whether catecholamines also occurred in fish brain and, if this were the case, to obtain data on their distribution.

Methods

Brains were prepared from a cyclostome (*Myxine glutinosa,* hagfish), an elasmobranch (*Squalus acanthias,* dogfish) and a teleost (*Gadus callarias,* cod) in freshly killed specimens. The brains of *Myxine* and *Gadus* were pooled in sufficiently large quantities to allow accurate estimations of the amines. After weighing, the brains were ground in a mortar with 10 per cent trichloroacetic acid, and filtered after 1/2 hour extraction. The residue

Table I. Adrenaline and noradrenaline in fish brains (total)

	n	Adr. µg/g	Noradr. µg/g	Per cent adr.
Myxine glutinosa (Cyclostome)	15	< 0.02	< 0.02	–
Squalus acanthias (Elasmobranch)	8	0.11	0.37	23
Gadus callarias (Teleost)	5	0.03	0.27	10

Table II. Adrenaline and noradrenaline in the brain of Squalus acanthias (dogfish) (6 animals)

Part of brain	Total weight g	Adr. µg/g	Noradr. µg/g	Adr. %
Telencephalon	5.2	0.12	0.49	20
Optic lobes	3.3	0.20	0.31	39
Diencephalon	0.77	0.31	0.55	36
Hypothalamus	0.62	0.33	0.44	43
Pituitary gland	0.38	0.41	1.5	22
Cerebellum	3.0	0.011	0.056	16
Medulla oblongata	3.8	0.13	0.25	34
Total brain (6 animals)	17.1	0.14	0.35	29
Total brain (2 animals)	4.4	0.072	0.39	16

was washed and the remaining solvent pressed out. The reaction was adjusted to pH 8.2—8.3 with 1 N Na OH and the catecholamines adsorbed on an alumina column, eluted with 0.25 N acetic acid and estimated fluorimetrically according to EULER and LISHAJKO (1959).

The brains of 6 specimens of *Squalus* were divided in 7 portions (telencephalon, optic lobes, diencephalon, hypothalamus, hypophysis, cerebellum and medulla oblongata), and extracted for catecholamines as described above.

Results

The results are given in Table I and II, showing the amounts of adrenaline and noradrenaline in the whole brain of the three types of fish studied, and their distribution in various parts of the brain of the dogfish.

As seen in Table I the amount of catecholamines is very small in the *Myxine* brain while it may be considered as relatively high in *Squalus* and *Gadus*, being of the same order of magnitude as in mammalian brain.

The relative amounts of noradrenaline and adrenaline also show characteristic differences in the different kinds of fish, adrenaline being relatively higher in *Squalus* than in the teleost studied.

Table II gives the absolute and relative amounts of adrenaline and noradrenaline in the different parts of the brain of *Squalus*. As seen in the table the catecholamine amounts are highest in the diencephalon, in hypothalamus, and in the hypophysis while they are particularly low in the cerebellum.

In Table II the adrenaline and noradrenaline content is also computed for the whole brain of the 6 animals. In addition, figures are given for 2 more animals from another series.

Discussion

The demonstration of fairly high amounts of catecholamines, chiefly noradrenaline, in different parts of the brain in elasmobranch and teleost fishes supports the idea that this hormone plays a part in the function of the brain. The small amounts found in the brain of *Myxine*, belonging to the primitive branch of cyclostomes, on the other hand, suggest that catecholamines enter as regulators of certain central nervous functions relatively late in the evolution chain. However, certain other organs of *Myxine*, particularly the heart, contain strikingly large amounts of catecholamines (Östlund *et al.* 1960, Euler and Fänge 1961).

The uneven distribution of noradrenaline in the brain of *Squalus* presents certain similarities to the characteristic distribution in mammals. Particularly noteworthy are the high amounts found in the hypothalamus, a sympathetic center in higher animals, and equally typical are the low quantities found in the cerebellum. The latter finding seems to indicate that noradrenaline has a specific function in certain parts of the brain and that this may, in principle, be the same in higher developed fishes as in mammals.

As to the mode of storage of catecholamines in the fish brain no data are as yet available. It may be assumed, however, that the relatively large amounts found, do not derive from postsynaptic sympathetic neurons but in all probability are stored, at least to a great part, in chromaffin cells. The presence of different kinds of chromaffin cells probably serving as stores of the catecholamines in the *Myxine* heart has recently been reported (Östlund *et al.* 1960).

This work was supported by grants from Gustaf och Tyra Svenssons Minne and from the Air Force Office of Scientific Research of the Air Research and Development Command, United States Air Force, through its European Office which is gratefully acknowledged. I am greatly indebted to Dr. B. Swedmark and Mr. Jørgen Lützen for valuable cooperation in the collection and preparation of material.

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Acta physiol. scand. 1961. 52. 65—74

From the Department of Medical Physiology, University of Copenhagen, Denmark

Studies on the Transfer of Phosphate from Mother to Foetus in the Rabbit

By

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Received 26 January 1961

Abstract

FUCHS, A.-R. and F. FUCHS. *Studies on the transfer of phosphate from mother to foetus in the rabbit.* Acta physiol. scand. 1961. *52*. 65—74. — The transfer of phosphate from mother to foetus was studied in rabbits with radioactive P. As in guinea pigs the inorganic phosphate concentration is higher in the foetal than in the maternal plasma, and the transfer takes place against the gradient. The transfer rates calculated from experiments of 30 min duration did only exceed or approach the rate of foetal P retention when the foetal and placental uptake rates were combined. In an experiment of 120 min duration the foetal uptake alone equalled the foetal retention. The combined foetal and placental uptake rates in experiments of varying duration were relatively constant.

In some of the rabbits gestation was artificially prolonged by daily injections of progesterone. The foetal P concentration increases up to but not beyond term. The placental concentration is fairly constant in the second half of gestation and after term, but goes up when intrauterine foetal death occurs, indicating that the placenta stays alive for some time and continues to take up phosphate. The active transport mechanism in the placenta must be localized to the chorionic cells, probably at their boundary toward the intervillous space, building up in the placenta a phosphate pool, from which the foetus is supplied.

In previous work on the placental transfer of phosphate in the guinea pig (FUCHS and FUCHS 1957 a, b, c, FUCHS 1957) we found that inorganic phosphate is transferred from the maternal to the foetal plasma across the placental barrier against a concentration gradient, and that the placental tissue contains considerable amounts of inorganic phosphate, forming a pool from which the foetus is supplied. The rates of phosphorus uptake in the foetus and in the placenta from the maternal plasma phosphate were measured with the aid of

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radioactive phosphorus, and it was found that these two rates had to be combined to cover the rate of foetal deposition of phosphorus, as determined chemically by ashing foetuses of different gestation ages. It was further found that there is some return of phosphate from the foetus to the mother, and that the safety factor, *i.e.* the ratio between the rate of foetal phosphorus uptake from maternal plasma phosphate and the rate of foetal phosphorus deposition, became very low, less than 1.5, at the end of gestation.

It was decided to carry out a series of experiments in the rabbit with the same technique in order to find out whether the placental transfer of phosphate follows the same pattern in this animal. In addition, it was found of interest to study the phosphate transfer in artificially prolonged gestation to see whether a placental insufficiency occurred after term. The results of the study of prolonged pregnancies have been discussed elsewhere (FUCHS and FUCHS 1960) and will only be briefly mentioned here.

Methods

Virginal Danish White Land Rabbits of uniform breed and of approximately the same age and weight were mated under supervision. From previous studies (FUCHS and FUCHS 1958) the average length of gestation was known to be around 31 days.

The rabbits to be studied after term were given 5 mg progesterone in oil intramuscularly from a gestation age of 29 days plus 6 to 12 hours and daily until the operation one to 6 days later. The main group of rabbits were not given progesterone. They were operated upon between the 18th day of gestation and term.

The transfer of phosphate to the foetuses was studied with the aid of radioactive phosphorus (P^{32}) which was injected in the form of inorganic phosphate with high specific activity into an ear vein of the mother animal. After a certain period of time, generally 30 min, abdominal hysterotomy was performed and the foetuses and the placentas including the subplacentas were removed. Care was taken to avoid blood loss from the umbilical vessels or the placenta. Usually four foetuses and four placentas were ashed individually for total phosphorus and radiophosphate determinations. When the litter contained more than four foetuses it was attempted to collect blood from the umbilical vein of the remaining foetuses. During the experiment a number of blood samples were withdrawn from the carotid artery of the mother to determine the rate of disappearance of radiophosphate from the plasma. The concentration of inorganic phosphate was determined in the maternal plasma in each experiment and in the foetal plasma when available. The determination of plasma phosphate, total foetal and placental phosphorus, and radioactive phosphorus were carried out as previously described (FUCHS and FUCHS 1957 a, FUCHS 1957).

The amount of inorganic phosphate transferred to the product of conception was calculated as previously described from the amount of radiophosphate found in the foetus and placenta at the end of the experiment and the average specific activity of maternal plasma phosphate during the period.

Results

The activities of the blood samples from different experiments were plotted in the same diagram by expressing the results as relative activities, 100 per cent denoting the activity of one ml of plasma immediately after injection. This

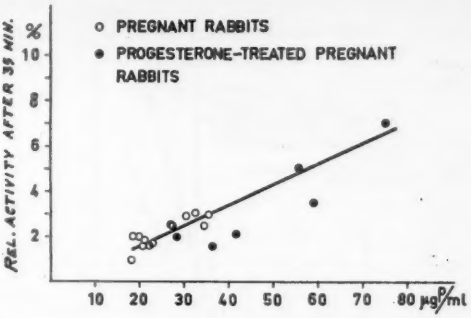

Fig. 1. Relation between the relative activity in plasma 35 min after intravenous injection of radioactive phosphate and the concentration of inorganic phosphorus in the plasma.

activity is calculated from the injected amount of radioactivity and the plasma volume, which according to COURTICE (1943) is 50 ml per kg body weight. When the disappearance curve from rabbits in the last half of pregnancy, obtained from the present experiments, is compared with values from non-pregnant rabbits, calculated from HEVESY and HAHN (1940), the rate of disappearance appears to be faster in the pregnant animals.

The concentration of inorganic phosphate in the maternal plasma varied between 18 and 36 µg P/ml in normal pregnancies. The disappearance rate was found to be inversely related to the concentration of inorganic phosphate. This is illustrated by Fig. 1, in which the relative activities 35 min after intravenous injection are plotted against the phosphate concentration. The lower the concentration the faster the disappearance, indicating a fairly constant turnover of the plasma phosphate. No relation could be found between the maternal plasma phosphate concentration and gestation age before term, but in the prolonged pregnancies the concentration increases significantly after the 32nd day. The foetal plasma concentration was determined in two cases, both on day 29, to 80 and 95 µg P/ml, respectively.

If pregnancy is prolonged, the foetal mortality increases for every day past term (CSAPO 1955, FUCHS and FUCHS 1958). In the present material 2 foetuses out of 10 were found dead in a litter removed 33 days after mating. At 34 days + 2 hours a whole litter of 6 were alive, whereas litters removed at 34 days + 18 hours and at 35 days, with 3, 5, and 9 foetuses, were all found dead in the uterus.

The average total phosphorus content of the foetuses and the average foetal weight with advancing gestation is shown in Fig. 2. From day 27 there are great variations, both within a litter and between the litters, and the size of the litter has a marked influence upon the average weight and phosphorus content of the foetuses. In prolonged pregnancies the foetuses continue to grow at approximately the same rate as at term, and they also increase their total phosphorus content.

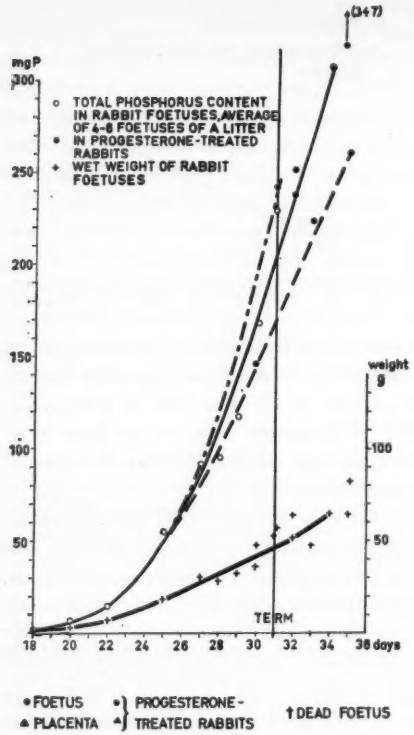

Fig. 2. Total phosphorus content and weight of rabbit foetuses at various stages of gestation. The broken lines indicate the variation of the litter averages during the last week of gestation.

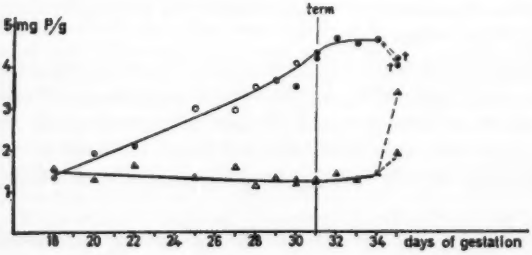

Fig. 3. Foetal and placental phosphorus content per g wet weight in the last half of gestation and in prolonged pregnancies.

If the mean foetal phosphorus concentration is considered (Fig. 3), it shows a steady increase until term, but after term the foetus does not get enough phosphorus to increase the concentration further. This finding is significant, because it was found that the foetal phosphorus concentration is very constant within a litter, regardless of its size, and depends only upon the gestation age.

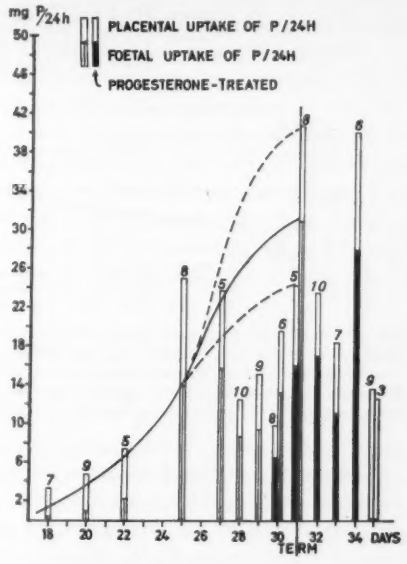

Fig. 4. Placental uptake (open columns) and foetal uptake (filled columns) of inorganic phosphate per 24 h as determined from radiophosphate experiments of 30 min duration, compared with the daily phosphorus retention in the foetus as calculated from Fig. 2. The solid line indicates the mean retention, the broken lines the limits of variation of litter averages. The numbers at the columns indicate litter size.

The weight of the blood-filled placentas was about 4 g at day 18 and increased until day 25, after which it remained fairly constant around 7 g. The placental phosphorus concentration remains almost constant during the whole second half of gestation and the first days after term. When the foetuses die, there is a sudden accumulation of phosphorus in the placentas, and consequently the concentration goes up (Fig. 3).

From the radiophosphate data the daily transfer of phosphate from mother to foetus can be calculated (Fig. 4). The lower part of each column shows the uptake per foetus which increases from 0.2 mg/24 h on day 18 to 30 mg/24 h at term. After term there is no further increase, the daily uptake seems to be fairly constant until the foetuses die. From day 27 the litter size has a marked influence upon the foetal phosphate uptake. The comparatively highest uptake per foetus is usually found in the smallest litters.

The rate of placental uptake of phosphate, indicated by the upper part of the columns in Fig. 4, is considerable already 18 days after mating, namely 3.2 mg/24 h as compared with the foetal uptake of 0.2 mg/24 h. The increase with advancing gestation is less, however, and occurs only until day 25, after which it remains fairly constant with an average of 7.4 mg/24 h. In the three cases with dead litters around day 35 foetal death appeared to have occurred a considerable period of time before in one case, and the partly separated placentas were not examined. In the two other cases the placentas appeared to be intact and the rate of uptake was as high as about 13 mg/24 h.

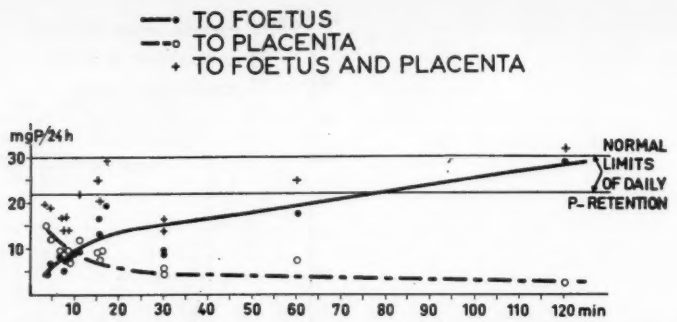

Fig. 5. Foetal and placental uptake of phosphate per 24 h, calculated from radiophosphate experiments of duration varying from 4 to 120 min, but all performed on day 29. The horizontal lines indicate the limits of foetal phosphorus retention per 24 h on this particular day of gestation. From the experiments of short duration the individual foetal and placental values are plotted, from those of 30—120 min duration only the averages.

The curves on Fig. 4 indicate the average daily phosphorus retention per foetus, as calculated from the data shown on Fig. 2. The broken lines show the variation limits of the litter averages and the solid line the calculated mean phosphorus retention. In the placentas no deposition of phosphorus takes place after day 25 except in case of intrauterine foetal death.

It is seen that the daily foetal uptake of phosphate as measured with radiophosphate in experiments of 30 min duration is definitely below the daily foetal phosphorus retention as measured by chemical analysis. This discrepancy is undoubtedly due to the presence of a placental phosphorus pool with which the phosphate becomes mixed during its passage from the maternal blood to the foetus. It is apparent that a better agreement with the foetal rate of retention is obtained when the foetal and placental rates of uptake are combined.

Further experiments were therefore carried out to elucidate the effect of the duration of the experiment on the calculated foetal and placental uptake of radiophosphate. Six rabbits were injected with radioactive phosphate at the same stage of gestation, 29 days after mating, but the foetuses and placentas were removed after different periods of time, varying from 4 to 120 min. The results, including the 30 min experiment on day 29 from the first series, are shown on Fig. 5. From the experiments of short duration the individual foetal and placental values are plotted, from those of 30 min and upwards only the averages. The calculated foetal rate of uptake is low in experiments of short duration, but increases to reach the rate of daily phosphorus retention in experiments of 2 hours duration. The placental rate of uptake, on the other hand, decreases with increasing duration of the experiment. The sum of the foetal and placental uptake rates was found to be fairly constant irrespective of the duration of the experiment, and of the same order of magnitude as the daily phosphorus retention.

The rapidly decreasing specific activity of the maternal plasma phosphate during the first minutes after intravenous injection of radiophosphate constitutes a considerable source of error in the calculations of the average specific activity during the experiment. This is probably the explanation of the failure of the combined foetal and placental uptake rates to reach the daily phosphorus retention in the very short experiments in Fig. 5. In some cases the failure may be attributed to a particularly large litter size; in such cases the rate of uptake per (foetus + placenta) might be expected to be below that in a case of average litter size. The two rabbits in Fig. 5 delivered after 30 min had 7 and 9 foetuses, respectively, as compared with 3—6 foetuses in the other litters in this series.

Discussion

The placental transfer of inorganic phosphate in the rabbit follows the same pattern as previously shown in the guinea pig. In both species the foetal rate of uptake of phosphate as measured with radiophosphate in experiments of 30 min duration is too low to account for the phosphorus retention actually taking place. In the guinea pig the addition of the placental uptake rate brought the total uptake of the product of conception considerably above the rate of phosphorus retention, the safety factor being about 8 in the middle of gestation and 1.5 at the end. In the rabbit the calculated foetal rate of uptake increases with increasing duration of the experiment and becomes equal to the phosphorus retention rate in 2 hours experiments. The combined foetal and placental uptake rates are independent of the duration of the experiment (at least up to 2 hours). This justifies the use of the combined uptake rates for the calculation of the transferred amounts of phosphate.

These results together with the fact that the placenta continues to take up phosphate from the maternal plasma and increases its phosphorus concentration after cessation of the foetal circulation indicate the presence of a phosphorus pool in the placenta of the rabbit. The same is the case in the guinea pig, in which we demonstrated a high placental concentration of inorganic phosphate (from 0.25 to 0.55 mg/g wet weight). Together with some labile organic phosphates it obtained a specific activity between that of the maternal and that of the foetal plasma phosphate. The active mechanism required for the up-hill transport of inorganic phosphate from the lower maternal to the higher foetal concentration of phosphate in the plasma must therefore be located to the chorionic cells, probably at their boundary toward the intervillous space. Bothwell *et al.* (1958) have found that also the placental uptake of iron in the rabbit is an active process independent of the foetus.

Our findings indicate that in the rabbit there is no safety margin for the transfer of phosphate in the second half of gestation. During the last four days before term the combined uptake rates even failed to reach the average phosphorus retention in most cases.

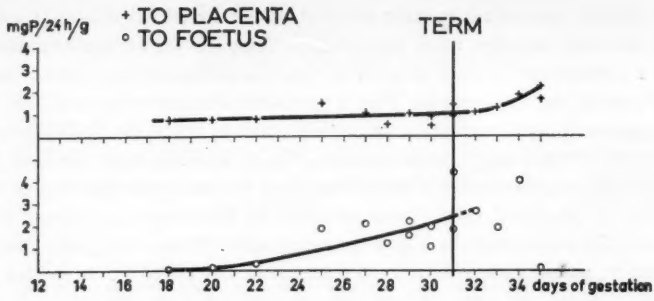

Fig. 6. Daily transfer of phosphate to foetus and placenta per g wet weight of the placenta at various stages of gestation, as calculated from radiophosphate experiments of 30 min duration.

If considerable amounts of radiophosphate return to the mother during the experiment, the calculated transfer rates would be too low, but the results shown in Fig. 5, where the combined uptake is the same in short and long experiments, speak against this possibility. Probably the explanation of the low rates of uptake observed at the end of gestation is partly that the four cases in question had large litters, namely 10, 9, 8, and 6 foetuses, respectively, and partly that certain errors are inherent in the determination of the average specific activity of the maternal plasma phosphate during the experimental period. The precipitous fall in the specific activity within the first minutes after the injection obviously gives rise to a considerable inaccuracy in the calculation of the average in experiments of very short duration. Experiments of 30 min duration should offer opportunities for calculation of a more reliable average, and a priori there is no reason to expect the error to vary with the stage of gestation. By our way of calculation of the average specific activity a variation of this kind may, however, be expected, since all calculations were based on the same standard disappearance curve, constructed from data of experiments performed at different stages of gestation. Now, with the increasing foetal uptake of phosphorus towards term the rate of disappearance of radiophosphate from the maternal plasma is probably greater at the end of gestation than that indicated by the standard curve. It is therefore likely that, by using the standard curve, the transfer rates have been somewhat underestimated in this part of gestation. We feel that this together with the occurrence of large litters, may well account for the fact that (foetal + placental) uptake rates below the retention rates were observed in the experiments covering this period.

Organic phosphorus compounds or other pathways may participate in the supply of phosphorus to the foetus, but granting the correctness of the above explanation of the low uptake rates towards term, it may be concluded that inorganic phosphate is transferred from the maternal plasma in sufficient

amounts to account for the foetal phosphorus deposition in most cases. At the end of gestation the foetuses may not always get enough and consequently develop at less than the optimal rate. This supposition is supported by the observed variations in foetal size which are much greater than the variations in the guinea pig.

If the rates of phosphate transfer to the foetuses and placentas are calculated per unit weight placenta, the results shown in Fig. 6 are obtained. Per gram placenta the foetal uptake increases from 0.05 mg/24 h at day 18 to about 2.5 mg/24 h at term. During this period the placental barrier becomes thinner and its surface increases. The volumes of maternal and foetal blood in the placenta undoubtedly increase, as was demonstrated in the guinea pig (Fuchs 1953), and probably also the blood flow. All these morphological and physiological factors facilitate the increase of the transfer. That the demands on the placenta are greater in the rabbit than in the guinea pig is illustrated by the fact that the ratio foetal weight to placental weight increases by a factor of 20 in the last 13 days of the rabbit gestation and only by a factor of 5 in the corresponding period of the much longer gestation of the guinea pig, the last 26 days.

The rabbit and the guinea pig both have hæmochorial placentas with three layers of tissue separating the foetal blood from the maternal blood stream, namely chorion, connective tissue in the villi, and foetal endothelium. The human placenta belongs to the same morphological group, and another similarity of the human being is the fact that the concentration of inorganic phosphate is higher in the foetal plasma than in the maternal plasma. It would seem likely that the placental transfer of phosphate follows the same pattern in the human as demonstrated in the rabbit and the guinea pig.

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Acta physiol. scand. 1961. 52. 75—89

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Influence of Local Temperature Changes in the Preoptic Area and Rostral Hypothalamus on the Regulation of Food and Water Intake

By

BENGT ANDERSSON and BÖRJE LARSSON

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Abstract

ANDERSSON, B. and B. LARSSON. *Influence of local temperature changes in the preoptic area and rostral hypothalamus on the regulation of food and water intake.* Acta physiol. scand. 1961. *52.* 75—89. — Local cooling of the preoptic area and rostral hypothalamus induced eating in the fed goat. After dehydration to the state of aphagia, cooling of this area inhibited the animal's urge to drink and induced eating. Although in a normal goat feeding practically stops when the rectal temperature exceeds 40° C (APPLEMAN and DELOUCHE 1958), local cooling of the preoptic area and the rostral hypothalamus induced the goat to eat hay at body temperatures above 41° C. Warming the same area inhibited eating in the hungry animal and induced the goat to drink large quantities of water.

After inactivation of the preoptic "heat loss centre" by proton irradiation in a goat, the animal became adipsic but continued to eat hay with a seemingly good appetite at body temperatures above 41° C. The anorexic effect of warming the preoptic area thus does not seem to be due to a direct thermal effect on the hypothalamic "appetite centre". The results provide direct evidence in favour of BROBECK's (1948) "thermostatic" theory of the regulation of food intake and justify the further extension of this theory to involve also the regulation of water intake.

Several investigations have indicated that food intake is regulated through the interaction of a "satiety centre" in the ventromedial hypothalamus and an "appetite centre" in the lateral hypothalamus (BROBECK 1955). Water intake

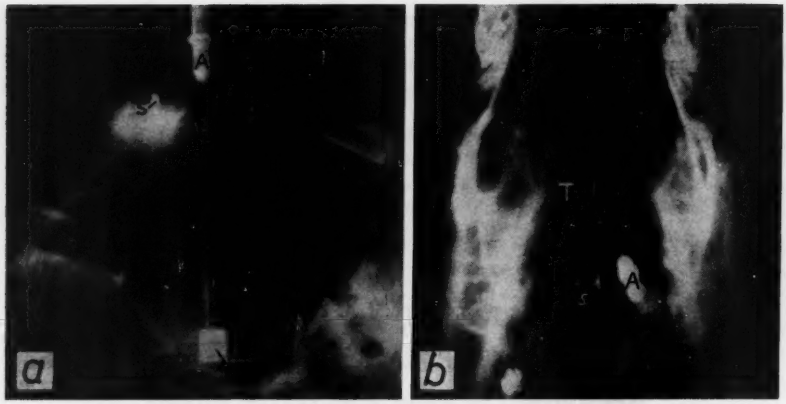

Fig. 1. *X-ray pictures of the head of the "thermode" goat taken from the side (a) and from above (b).*
T: The body of the thermode implanted medially in the preoptic area and rostral hypothalamus.
A: The needle applicator used for measurements of brain temperature close to the surface of the thermode.
s: Dental root screws used for fixation of dental cement to the skull bone.

seems to be regulated from parts of the hypothalamus partly overlapping the "satiety centre" and located medially and somewhat rostral to the "appetite centre" (ANDERSSON and MCCANN 1955). Cellular dehydration has been considered the main factor stimulating the hypothalamic "drinking centre" (WOLF 1950, ANDERSSON 1952). The changes in the internal environment of importance for the regulation of food intake may however be multiple. A theory of a glucostatic mechanism regulating food intake has been introduced (MAYER 1952), but adjustments of feeding may also be made in relation to the amount of stored fat in the body (KENNEDY 1953). BROBECK (1948, 1960) has stressed the intimate correlation between body temperature and food intake and has presented a "thermostatic" theory. He suggests that in inhibiting food intake, heat acts either upon heat sensitive neurons of the rostral hypothalamus and the preoptic area or directly upon neurons of the "appetite centre".

The experiments reported here provide evidence in favour of BROBECK's suggestion that a rise of the body temperature may inhibit food intake by the influence of heat sensitive neurons in the preoptic area and rostral hypothalamus. They also indicate that the hypothalamic "satiety centre" may be inhibited by the influence of cold sensitive elements in the same area and further that thermosensitive neurons in this part of the brain influence water intake in the reverse manner that they influenced food intake.

A further study of the effect on food and water intake and on temperature-regulation of lesions of various size in the preoptic area will be presented in a following publication (ANDERSSON, LARSSON and REXED 1961).

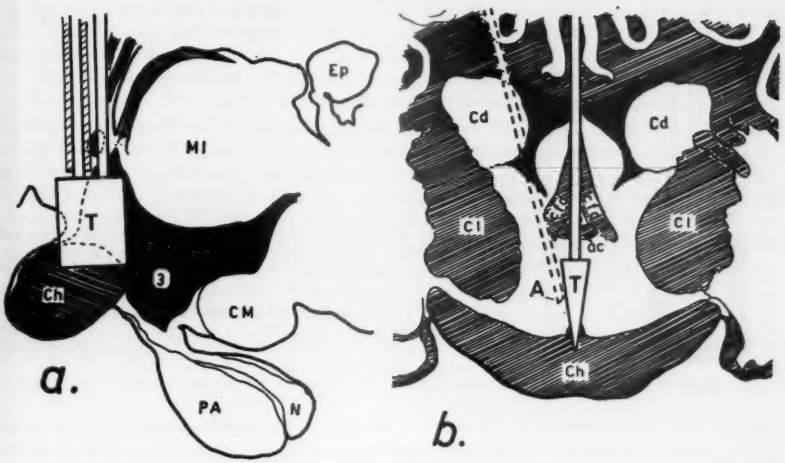

Fig. 2. *Drawings of a medial sagittal section through the hypothalamus (a) and of a transverse section through the preoptic area of the goat (b) to show the positions of the thermode (T) and the needle applicator (A).*

ac: Commissura anterior
Cd: Nucleus caudatus
cfd: Columna fornicis descendens
Ch: Chiasma opticum
CI: Capsula interna
CM: Corpus mammillare
Ep: Epiphysis
MI: Massa intermedia
N: Neurohypophysis
PA: Adenohypophysis
3: Third ventricle

Methods

The experiments were made in two goats. One animal had a silver thermode implanted medically in the preoptic area and rostral hypothalamus and the other goat had had its preoptic "heat loss centre" destroyed by proton irradiation. The principles for the use of the high-energy proton beam as a neurosurgical tool was described earlier (Larsson *et al.* 1958, Leksell *et al.* 1960). In the present animal permanent platinum iridium electrodes, previously implanted into the "heat loss centre" had been used as points of aim for the irradiation. This technique will be described elsewhere (Larsson and Andersson 1961).

Technique for implantation of a silver thermode into the brain stem: The technique used was similar to that previously described for implantation of permanent electrodes into the hypothalamus of horned goats (Andersson, Persson and Ström 1960 a). With the animal generally anesthetized, a holder with rods for guidance of the thermode and a needle applicator for brain temperature recording was temporarily screwed onto the surface of the skull bone. The correct placement of the holder was facilitated by previous X-ray studies and measurements on the skull. An opening in the skull bone, fitting the thermode, was then made along the midline and a smaller hole for the needle applicator was drilled through the bone more laterally. The dura mater was split along the midline of the brain to allow the introduction of the thermode into the brain. When the thermode and the needle applicator had been placed in the positions shown in Fig. 1 and 2, they

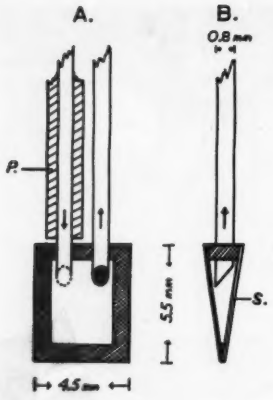

Fig. 3. *Drawings of the silver thermode used for cooling and warming of the preoptic area and the rostral hypothalamus.*
A: Lateral view
B: Frontal view
P: Polethylene tubing used as insulation of the inwards leading cannula.
S: The side walls of the thermode consisting of 0.2 mm thick silver plates.

were permanently attached to the bone of the skull with dental cement filling the holes in the bone. The dental cement was further secured to the bone by the help of dental root screws. The holder used for temporary fixation and guidance of the thermode was then removed, leaving only the two cannulas of the thermode and the base of the needle applicator sticking up on the dorsal surface of the skull. The skin incision was sutured round the protruding layer of dental cement. Polyethylene tubing connected to the cannulas of the thermode and the leads from the needle applicator were fixed to one horn of the animal and the whole equipment was protected from external damage in the manner previously described (ANDERSSON *et al.* 1960 a). The kind of thermode used is shown in Fig. 3.

Technique of central cooling: When used for cooling the thermode was perfused via a 1.5 m long rubber tubing connecting a pressure flask, containing iced water, with the polyethylene tubing attached to the inwards leading, isolated cannula of the thermode. The end of the rubber tubing was hereby fixed on one horn. An applicator ("Ellab", type KC#1) was placed in the end of the rubber tubing allowing measurements of the temperature of the perfusion water at the junction of the rubber tubing and the polyethylene tubing on the horn. Since the animal was kept in its usual environment (collared in a metabolism cage) during all experiments, it was necessary to have a lead of this length to allow it full freedom to move as it was used to. The long rubber tubing, however, had the disadvantage that the temperature of the perfusion water entering the polyethylene tubing was as high as 19 to 20° C when the perfusion was performed in the usual environmental temperature of the goat (22° C). Perfusions made in the above described manner lowered the temperature at the surface of the thermode 9 to 10° C. The latency time from beginning of perfusion with cold water to maximal lowering of the brain temperature at the surface of the thermode was of the order of 50 secs to one minute. When the perfusion was suddenly stopped, it took one to one and a half minute for the temperature close to the surface of the thermode to reach blood temperature level again.

The cooling experiments reported here were all performed in the manner just described. However, by using shorter tubing connecting the pressure flask with the thermode and by having the goat placed in a colder environment, it was possible in other experiments to obtain a more intense, central cooling with principally the same technique.

Technique of central warming: All experiments involving central warming were also performed in an external temperature of 22° C. To obtain a reasonably high temperature

of the perfusion water, although using a long lead, an extra outlet for the warm water had to be fitted to the rubber tubing close to the head of the goat. Hot tap water (65° C) was used for perfusion and the perfusion pressure was kept close to 200 mm Hg. Due to heat losses in the rubber tubing, the temperature of the water at the junction on the horn was only of the order of 55° C. Identical perfusion technique was used in all experiments and was found to cause a temperature rise of about 8° C at the external surface of the thermode. The latency time from start of perfusion with warm water until the temperature at the surface of the thermode reached its maximum was about one minute. Thirty secs after a sudden stop of the perfusion, the temperature at the surface of the thermode had again fallen to the level of blood temperature.

Temperature recordings: Brain temperature at the surface of the thermode was recorded by connecting the leads from the implanted needle applicator ("Ellab", type K 8) to an "Ellab" Electric Universal Thermometer (type TE 3).

Blood temperature was recorded in a similar manner using an "Ellab" applicator (type F 6) inserted through the jugular vein into the superior vena cava at a distance of 5 to 10 cm from the heart.

Ear surface temperature, as an index of peripheral vasodilatation and vasoconstriction, was measured by the help of an "Ellab" surface applicator (type E 5) attached to the dorsal surface of one ear near its tip.

Rectal temperature was measured occasionally during the experiments using a mercury thermometer.

At times when the thermode was not perfused, no or very small differences were observed between blood and brain temperature. Rectal temperature, however, was found to be a poor index of real body temperature, since it lagged considerably behind relatively rapid changes in blood and brain temperature.

Recordings of ruminal motility: The animals were supplied with closed ruminal fistulas and ruminal motility was occasionally recorded as previously described (Andersson, Kitchell and Persson 1959).

Recordings of respiratory rate were made as previously described (Andersson, Persson and Ström 1960 b).

Care of the animals: The goats were constantly kept collared in metabolism cages and had for more than a month after the operations become used to the experimental conditions. They were placed in a room where the temperature was kept at 22° C and were fed regularly at 9 o'clock a. m. They then obtained 250 g groats with 4 g NaCl added and 2 kg hay. The groats was usually consumed during 15 to 20 minutes whereas the hay ration lasted till in the afternoon. Some less palatable residuals of the hay ration were usually left on the bottom of the foddering-rack till next feeding time and were then discarded.

All observations concerning food intake reported here concern the animals' consumption of hay.

With the exception of certain periods of purposely induced water deprivation, the goats had always free access to water. The water container was placed at the side of the foddering rack in front of the goats. Daily water intake and urinary output were recorded.

Results

Localization of the thermode and approximate degrees and extent of central cooling and warming

Since the "thermode" goat is still alive and is used for further experiments, no histological localization of the site of the thermode has as yet been made.

However, the study of X-ray pictures of the head of the animal and similar studies on the sagittally split heads of dead animals reveals that the position of the thermode and the needle applicator is medially in the rostral hypothalamus and the preoptic area as shown in Fig. 2 a and b.

More difficult to judge is the temperature gradients in the brain tissue a round the thermode during perfusion with cold or warm water. Measurements of brain temperature were made in a control animal with an identical thermode implanted somewhat more rostrally and with a needle applicator for temperature recording placed 6 mm lateral to the lateral surface of the thermode. The measurements revealed that the same technique of cooling which caused a drop of the temperature close to the surface of the thermode of 9 to 10° C, resulted in a temperature drop of 1 to 1 1/2° C 6 mm lateral to the thermode. Provided the main flow of blood was directed rostrally, the shape of the thermode (Fig. 3) may justify the assumption that the temperature gradient in the posterior direction (towards the hypothalamus) was much steeper than that lateral to the thermode.

When the goat had a normal blood and brain temperature (39.0° to 39.5° C) the perfusion with cold water in the manner described above caused the temperature close to the surface of the thermode to fall to between 29 and 30° C. The perfusion with warm water caused a rise of the temperature at the same place to 48 to 49° C.

Effects of cooling the preoptic area and rostral hypothalamus

a) *Thermoregulatory effects*

The thermoregulatory effects of local cooling in the preoptic area and the rostral hypothalamus will be described separately (ANDERSSON 1961). In this connection it may be sufficient to mention that cooling induced shivering and peripheral vasoconstriction. When the local cooling was performed for longer periods of time, this mobilization of cold defense mechanisms led to a considerable rise of body temperature. If under such circumstances central cooling was stopped, heat loss mechanisms were activated which worked to put the body temperature of the animal back to normal level (Fig. 4).

b) *Alimentary effects*

The fed animal with free access to water: Cooling of the preoptic area and rostral hypothalamus was made on several occasions three to five hours after the goat had been fed. The animal had generally free access to hay but was either not eating any longer, or was only occasionally taking a few straws when the experiments started. Within two to three minutes of central cooling, however, it started to eat hay with a seemingly good appetite, stopping again to eat one to two minutes after cessation of central cooling.

On a few occasions when central cooling was performed in the fed animal with no access to hay, it was seen to induce rumination.

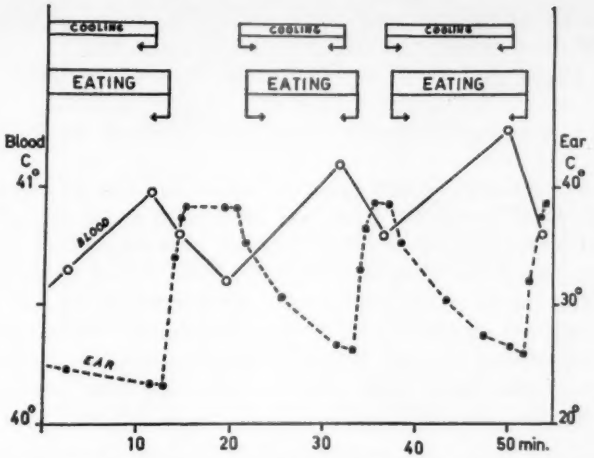

Fig. 4. *The second part of an experiment in which the preoptic area and rostral hypothalamus was repeatedly cooled.*

The goat had free access to hay during the experiment which started at normal feeding time. The increasing blood temperature during the periods of central cooling was due to shivering and peripheral vasoconstriction. During the intervals between periods of central cooling, heat loss mechanisms were mobilized, which worked to put the body temperature back to a normal level. The animal was eating hay with a seemingly good appetite during the periods of central cooling, but stopped eating simultaneously to the onset of peripheral vasodilatation (rise of ear temperature) following upon discontinuation of central cooling. Towards the end of the experiment central cooling induced eating in spite of a blood temperature well above 41° C.

The non-fed animal with free access to water: APPLEMAN and DELOUCHE (1958) found in the normal goat that feeding practically stops when rectal temperature exceeds 40° C. It therefore seemed to be of interest to find out whether or not the present animal would eat hay at still higher body temperatures when its preoptic area and rostral hypothalamus was cooled, and further to try to determine the body temperature at which the goat would stop eating when no longer centrally cooled. These experiments started at regular feeding time. The second part of such an experiment is shown in Fig. 4. The rectal temperature was 39.2° C and the blood and brain temperature 39.6° C when this experiment started. During the first 10 min of central cooling the goat had no access to hay. Cooling of the preoptic area and rostral hypothalamus was then found to increase the strength and the frequency of ruminal contractions. When later given hay the animal started to eat with a good appetite. During the first interruption of central cooling the goat continued to eat, but with diminished intensity. The blood temperature was then 40.2° C. During the second interruption of central cooling, when blood temperature had reached 40.6° C, the goat stopped eating but started again during the following period of central cooling. As is shown in

Fig. 4, intense eating of hay could still be induced by cooling the preoptic area and rostral hypothalamus at blood temperatures above 41.2° C.

Eating generally started 30 sec to 1 1/2 min after commencement of cooling and continued for one to two minutes after cessation of cooling. Eating was then seen to stop simultaneously to the onset of peripheral vasodilatation (Fig. 4).

The water deprivated goat with free access to food: On two occasions the goat was not given any water for three days but was fed its usual ration. On both occasions it stopped eating on the third day of water deprivation and was then very eager to drink minute amounts of water offered. Two or three minutes after beginning of central cooling, however, it no longer showed any interest in offered water, but instead started to eat accessible hay. One minute after the cooling was stopped the goat turned its attention from the hay and started to drink large amounts of water from the water container, which had been filled during the period of central cooling.

c) *Other effects of cooling the preoptic area and rostral hypothalamus*

During the 24 hours following experiments in which the preoptic area and rostral hypothalamus had been cooled for longer periods the goat was found to have passed large amounts of diluted urine and to have drunk about three times the normal amount of water. This phenomenon will be the subject of further studies.

Effects of warming the preoptic area and rostral hypothalamus

a) *Thermoregulatory effects*

As was expected from the results of earlier investigations (MAGOUN *et al.* 1938 and others), warming the preoptic area and rostral hypothalamus had the reverse thermoregulatory effect to that of cooling. The perfusion of the thermode with warm water, causing a rise of the temperature at its external surface to 48 to 49° C, resulted in polypnea and peripheral vasodilatation (Fig. 5) and thus led to a fall of the body temperature.

b) *Alimentary effects*

The experiments in which local warming of the preoptic area and the rostral hypothalamus was performed, started at regular feeding time when the goat had just obtained its hay ration and had started to eat with a good appetite. On most occasions the animal had free access to water, having the filled water container placed in the usual manner. Thirty secs to one minute after commencement of central warming the goat stopped eating. The brain temperature close to the surface of the thermode was then of the order of 43 to 46° C. Within a minute after the animal had stopped eating it showed obvious signs of intense thirst. If no water was accessible it started to lick the drops of water coming out of the outlet tubing of the thermode, but no longer showed any interest in

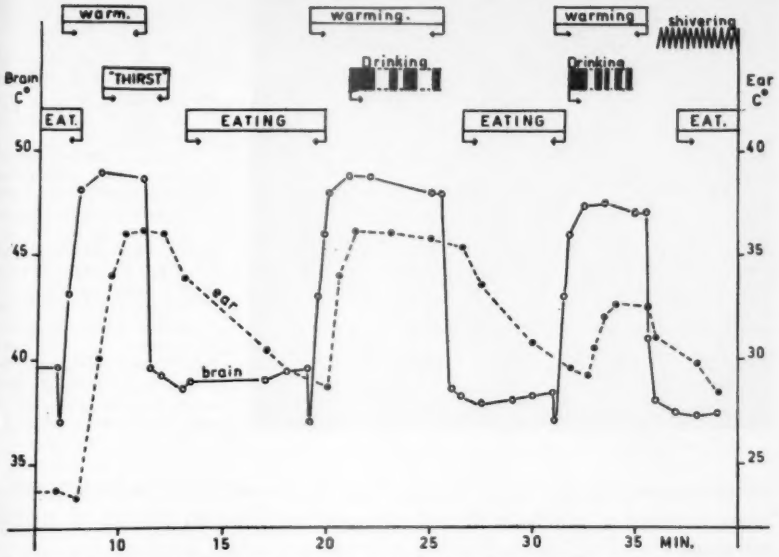

Fig. 5. *Results of warming the preoptic area and rostral hypothalamus in the previously hungry animal.* Brain temperature was recorded close to the surface of the thermode. The goat was fed hay at the beginning of the experiment and had free access to water except during the first period of central warming. During the periods of warming eating stopped simultaneously to the onset, of peripheral vasodilatation (rise of ear temperature), and started again when ear surface temperature had begun to fall after discontinuation of central warming. The perfusion of the thermode with warm water induced a strong urge to drink. During the first period of central warming, when the water container was temporarily removed, it was evidenced by the animal's licking of the drops of water coming out of the outlet tubing of the thermode ("thirst") and later on by the repeated drinking of large amounts of water during the periods of central warming.

water a minute after central warming was discontinued. If water was accessible during warming of the preoptic area and rostral hypothalamus the goat turned to the water container and started to drink. It continued to drink large amounts of water in one sequence. If central warming was not interrupted, the goat, after the first long period of drinking, continued to hold its head over the water container, and now and then drank again for shorter periods of time (Fig. 5). During intervals between periods of central warming the goat, however, refused to drink. Fig. 5 shows the time sequence of the periods of eating and drinking during and between repeated periods of central warming. In this experiment the goat drank 3.9 l of water at 18° C due to warming of the preoptic area and rostral hypothalamus. As can be seen in the figure there was a close correlation in time between stopping eating, the onset of peripheral vasodilatation and the start of drinking. The repeated mobilization of heat loss mechanisms and the drinking of large amounts of relatively cold water gradually lowered the body

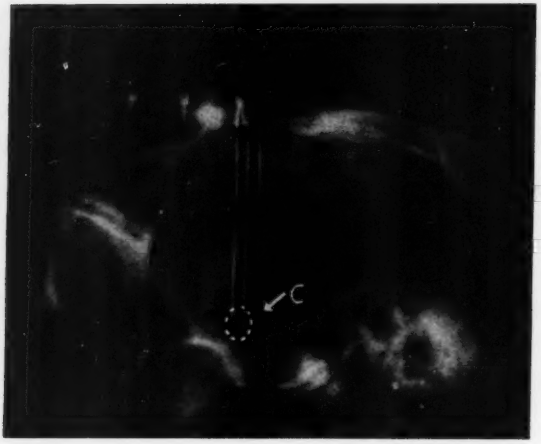

Fig. 6. *An X-ray picture of the head of the goat which had had its preoptic "heat loss centre" permanently inactivated by proton irradiation.*

The tips of the two rostral electrodes were implanted into the dorsal part of the "heat loss centre". The encircled area marks the approximate sagittal extent of the irradiated area. The tip of the posterior electrode (C) was situated outside the irradiated area. Electrical stimulation via this electrode caused shivering. Prolonged stimulation resulted in a considerable rise of body temperature (Fig. 7).

temperature of the goat more than 2° C which, towards the end of the experiment, resulted in shivering during the intervals between periods of central warming.

Experiments in a goat in which the preoptic "heat loss centre" was permanently inactivated

In a series of goats, having permanent electrodes implanted in the preoptic "heat loss centre", the tips of the electrodes were used as points of aim for proton irradiation of this "centre" (LARSSON and ANDERSSON 1961). The effect of the subsequent lesions on food and water intake and on temperature regulation was studied and will be described elsewhere (ANDERSSON *et al.* 1961). Three animals belonging to this series developed permanent adipsia, in two the adipsia was accompanied by hypophagia or aphagia. The third goat, however, retained its urge to eat to an almost normal extent. This goat had one electrode placed slightly dorsal and posterior to the irradiated part of the brain stem (Fig. 6 C). Electrical stimulation via this electrode caused shivering and by continuing the stimulation the body temperature of the animal could be increased more than 2° C. The complete inactivation of the "heat loss centre" was evidenced by the fact that the animal no longer reacted with peripheral vasodilatation and polypnea during hyperthermia. Stopping stimulation when the animal's body temperature had reached 41.5° C was thus not followed by any increase in the ear surface temperature or any increased respiratory rate (Fig. 7). Of special interest in this connection was the observation that the goat retained its urge to eat hay with a seemingly good appetite even at this very high level of body temperature (Fig. 7).

No histological examination of the preoptic lesion has so far been made in

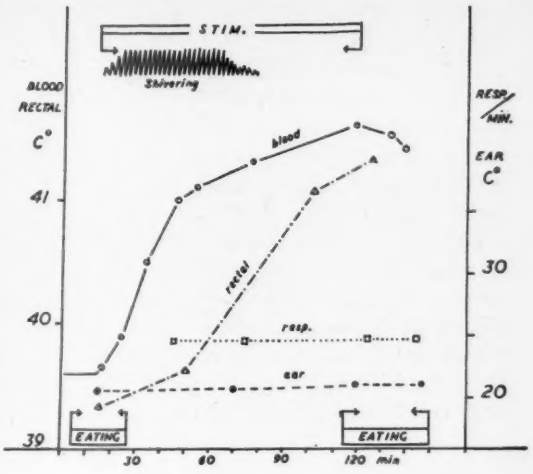

Fig. 7. *Results of electrical stimulation via electrode C in the goat which had had its preoptic "heat loss centre" permanently inactivated.*

The prolonged stimulation (stim.) caused shivering which led to a 2° C increase in body temperature. The total absence of central regulation against hyperthermia was evidenced by the lack of polypnea (low respiratory rate) and by remaining peripheral vasoconstriction (unchanged ear surface temperature) after discontinuation of the stimulation. During the periods marked "eating" the animal was offered hay and was eating with a good appetite. During the second period it was eating as intensively as before in spite of a body temperature well above 41° C.

this animal, but the extent of the area subjected to irradiation is indicated in Fig. 6. The irradiation did not involve any part of the hypothalamic "feeding centre".

Even in another respect the regulation of food intake was in the irradiated animal different from that of a normal goat. To test the completeness of its adipsia the goat was on three occasions not given water via the ruminal fistula for three or four days. Unlike a normal goat it continued to eat its daily ration of food even on the fourth day of water deprivation. The degree of dehydration was then very severe (Plasma Na: 174 meq/l; Plasma Cl: 134 meq/l).

Discussion

The present series of experiments was started because it had been observed that lesions in the preoptic area, intentionally made to destroy the "heat loss centre", caused permanent adipsia and in cases with larger lesions in addition hypophagia or even aphagia (ANDERSSON *et al.* 1961). These observations led to the assumption that the central projections from the hypothalamic "feeding" and "drinking centres" pass via the preoptic area to the rhinencephalon and that these connections are essential for the development of a conscious urge to eat and to drink. The first experiment in which alimentary effects of central cooling were studied, was therefore performed as an attempt to cause a cold block of the central projections from the hypothalamic "drinking centre" in the thirsty goat. For this reason the animal had been deprivated of water for

three days and was no longer eating the food offered. Cooling was found to inhibit the animal's urge to drink but in addition it also induced eating. This finding indicated a close interaction between thermosensitive elements in the preoptic area and rostral hypothalamus and the hypothalamic "feeding centre", as suggested by BROBECK (1960). The animal, being "basically hungry", might however have started to eat because it was released from the uncomfortable sensation of strong thirst. Similar experiments were therefore made when the goat had been kept with free access to water. The experiments were also extended to studies of alimentary effects due to local warming of the preoptic area and the rostral hypothalamus. It was found that central cooling induced eating in the fed goat, also, and that the animal could be induced to eat hay with a seemingly good appetite in spite of a body temperature much higher than that at which a normal goat stops eating (APPLEMAN and DELOUCHE 1958). Local warming of the preoptic area and the rostral hypothalamus had the reverse effect. It inhibited eating in the previously hungry animal and induced it to drink large quantities of water, although not having shown any signs of thirst the moment before local warming started.

The short latencies of the alimentary effects of central cooling and warming and the reversibility of these effects makes it unlikely that they should be due to chemical changes of the internal environment which have a direct stimulatory or inhibitory effect on the hypothalamic "feeding" and "drinking centres". It seems more probable that the changed activity in these "centres" may have been due to a neural influence. The close time relation between the start of eating and the onset of peripheral vasoconstriction and between stop of eating and the onset of peripheral vasodilatation is in evidence of an intimate functional correlation between a thermoregulatory mechanism and the hypothalamic "feeding centre". This is entirely in line with BROBECK's "thermostatic" theory as illustrated by the following quotation from his recent review "Food and Temperature" (1960):

"The possibility that the necessary brake (satiety) is applied by the heat, itself, was proposed first by BOOTH and STRANG (1936), after observations had been made on normal and obese human subjects. They recorded the elevation of skin temperature which follows a meal and attempted to correlate this elevation with the onset of satiety. In their view, the body appreciates the sensation of warmth in the skin, and this leads in turn to a feeling of comfort and satiation. It now appears more likely that the appreciation is not peripheral, but central ... that the SDA (specific dynamic action) acts directly upon cells in or just ahead of the hypothalamus to evoke cutaneous vasodilatation, and that this is accompanied by central inhibition of appetite and induction of satiety."

Since MAGOUN *et al.* (1938) had shown that local warming of the preoptic area causes the mobilization of various heat loss mechanisms, this part of the brain has been considered to be the site of a "heat loss centre", which is exclusively concerned with the regulation against hyperthermia. More recent

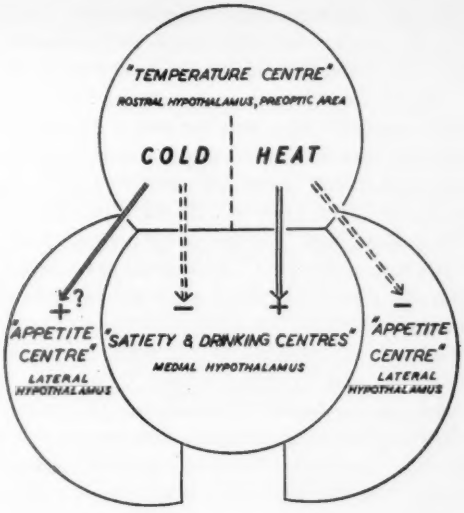

Fig. 8. *A tentative explanation of the alimentary effects of cooling and warming the preoptic area and rostral hypothalamus.*

The suggested explanation of the effect of warming on food intake is in accordance with BROBECK's (1948, 1960) "thermostatic" theory.

studies have however shown that local cooling within (KUNDT, BRÜCK and HENSEL 1957, ANDERSSON 1961) and electrical stimulation in the close vicinity of (ANDERSSON 1957) the same area induce shivering and peripheral vasoconstriction. The latter findings indicate that this part of the brain is not only the site of a heat loss mechanism, but is also involved in the defense against hypothermia.

Since cooling of the preoptic area and the rostral hypothalamus induced eating in the fed, normothermic animal, it may be assumed that the stimulation of a pre- and supraoptic cold defense mechanism activates the hypothalamic "appetite centre" either directly or by inhibiting the ventromedial "satiety centre". The latter may be the most probable explanation since cooling at the same time inhibited the animal's urge to drink and the "drinking centre" is anatomically closely related to the "satiety centre" (STEVENSON, WELT and ORLOFF 1950, ANDERSSON and MCCANN 1955).

Cellular dehydration has been considered to be the main factor eliciting the sensation of thirst (WOLF 1950, 1958). But the strong urge to drink, induced in the "thermode" animal by warming the preoptic area and the rostral hypothalamus is not likely to have involved any change in the osmolarity of the internal environment. The urge to drink disappeared soon after discontinuation of central warming and rapidly returned when central warming was started again (Fig. 5). It may therefore be easier to explain the drinking effect as due to a stimulatory action exerted from the preoptic "heat loss centre" on a hypothalamic mechanism concerned with the regulation of water intake. To judge from the present findings, the activation of a central mechanism concerned

with the regulation against hypothermia, would have the reverse effect on the hypothalamic "drinking centre". In summary the results reported here may thus be tentatively explained in the manner shown in Fig. 8.

One main objection to this explanation is that the cooling and the warming of the preoptic area and the rostral hypothalamus may have affected directly the areas of the hypothalamus concerned with the regulation of food and water intake. A spread of the local warming and cooling to the lateral "appetite centre" seems unlikely, however. In addition, the goat which had had the preoptic "heat loss centre" destroyed by proton irradiation, did eat at body temperatures well above 41° C. This seems to exclude the possibility that local warming has any direct inhibitory effect on the activity of the hypothalamic "appetite centre". Presently, however, it can not be ruled out that the perfusion of the thermode with warm and cold water may have changed the temperature in the "satiety" and "drinking centres", especially since the latter was found to extend into the rostral hypothalamus (ANDERSSON and MCCANN 1955). Provided the local warming acted as a non-specific stimulus to the neurons and the local cooling directly inhibited all neuronal activity within the affected area of the brain, the observed alimentary effects could be explained as being due to a spread of heat and cold to the "satiety" and "drinking centres". Such an explanation seems unlikely, since the perfusion of the thermode with warm water was only seen to induce thermoregulatory and alimentary effects. Had the local warming acted as a non-specific stimulus to all neurons within the heated area, it probably would have caused a much more complex and diffuse reaction in the animal.

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A Criticism of the Carcass Analysis Procedure for the Determination of Amino Acid Requirements

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Abstract

ERICSON, L.-E. *A criticism of the carcass analysis procedure for the determination of amino acid requirements.* Acta physiol. scand. 1961. *52.* 90—98. — The carcass analysis procedure is examined as a method for the determination of amino acid requirements. Using a simple reaction sequence to illustrate the fate of an amino acid in the body, enzyme kinetics are applied to demonstrate the impossibility of estimating the requirement of one compound in relation to another from knowledge of only the concentrations of the two compounds in the organism. The further complications arising from (a) the known individual variations in the concentrations of enzymes in the body, (b) the utilization by the organism of certain essential amino acids for purposes other than protein synthesis, (c) the partial ability of the organism to synthesize some essential amino acids, (d) the different rates of breakdown and excretion of the amino acids and (e) the fact that the body consists not of one but of a large number of different proteins with varying metabolic stability are briefly discussed. Some experimental data are cited which indicate that the carcass analysis procedure can give requirement values that are greatly erroneous. The criticism is presented with the hope that it will stimulate further theoretical and experimental studies in order to define the limitations and possibilities of the method.

In discussions concerning the amino acid requirements of higher animals the opinion is often expressed that these requirements are reflected by the amino acid composition of the animal under study (BEACH, MUNKS and ROBINSON 1943, MUNKS *et al.* 1945, BOCOBO *et al.* 1952, WILLIAMS *et al.* 1954, SHAR-

PENAK 1958, ALBANESE 1955, 1959, MITCHELL 1959). It is stated, that if the requirement of *one* amino acid is known, the body's need for all the other amino acids can be calculated from its amino acid composition. In other words, the *ratios of the requirements* of the different essential amino acids should be identical with the *ratios of the concentrations* of these amino acids in the animal body. This method of determining the amino acid requirements from body composition data is generally referred to as the carcass analysis procedure.

Various animal species have a remarkably similar amino acid composition (*cf.* BEACH, MUNKS and ROBINSON 1943, BOCOBO *et al.* 1952, WILLIAMS *et al.* 1954, MITCHELL 1959). This would make the caracass analysis procedure more useful as the amino acid composition of one kind of animal *e. g.* the rat, could be used for calculating the amino acid requirements of another kind of animal, *e. g.* the human. It is also postulated, that since muscle protein represents nearly three quarters of the total protein of higher animals, it should be sufficient to know the amino acid composition of this tissue in order to estimate the desirable ratios of dietary amino acids. This approximation seems to be supported by the isotope experiments of SCHÖNHEIMER *et al.* (1949) and others who have shown, that of the total quantity of protein in the body that is exchanged per unit time, muscle protein provides about sixty-five per cent.[1] Similarly, when protein is lost from the body during a period of protein starvation, most of it is derived from muscle tissue (ADDIS, POO and LEW, 1936). It may therefore be justifiable to consider, as some authors have done (*cf.* ALBANESE 1955, 1959, SHARPENAK 1958, MITCHELL 1959), only the composition of the muscle protein when estimating amino acid requirements by the carcass analysis method. What may not be correct, however, is the basic assumption that the composition of a tissue permits a prediction of the need for maintenance or growth of this tissue.

Simplified enzyme kinetic consideration

All organic constituents of the food undergo a series of reactions in the organism before being excreted. A simple reaction sequence that could illustrate this fact and serve as a model for enzyme kinetic considerations, would be one in which a compound (1) is first in one step converted to the form in which it is normally found in the cell (2) and thereafter in one further step metabolized to the form (3) in which it is excreted:

$$(1) \rightarrow (2) \rightarrow (3)$$

It is assumed here that in these irreversible convertions no other substance participates, apart from two enzymes catalyzing the reactions. We can therefore write:

$$(1) \qquad A_1 + E_1 \underset{k_2}{\overset{k_1}{\rightleftarrows}} C_1 \xrightarrow{k_3} E_1 + A_2; \qquad A_2 + E_2 \underset{k_5}{\overset{k_4}{\rightleftarrows}} C_2 \xrightarrow{k_6} E_2 + A_3$$

where

[1] The difficulty in finding the correct interpretation for data obtained in isotope studies of the type performed by Schönheimer and others should be mentioned.

A_1 = the form in which the compound is supplied.
A_2 = the form in which the compound appears in the cell after having been transformed by the enzyme E_1.
A_3 = the form in which the compound is excreted after having been further converted by the enzyme E_2.
C_1 and C_2 = the enzyme-substrate complexes formed when A_1 reacts with E_1 and A_2 with E_2.
k_1—k_6 = rate constants.

Letting the symbols represent both the type of compound and the concentration (REINER 1959) we can write the rate equations for A_1, C_1, A_2, C_2 and A_3:

$$\frac{dA_1}{dt} = -k_1 \cdot A_1 \cdot E_1 + k_2 \cdot C_1 \tag{2}$$

$$\frac{dC_1}{dt} = k_1 \cdot A_1 \cdot E_1 - k_2 \cdot C_1 - k_3 \cdot C_1 \tag{3}$$

$$\frac{dA_2}{dt} = k_3 \cdot C_1 - k_4 \cdot A_2 \cdot E_2 + k_5 \cdot C_2 \tag{4}$$

$$\frac{dC_2}{dt} = k_4 \cdot A_2 \cdot E_2 - k_5 \cdot C_2 - k_6 \cdot C_2 \tag{5}$$

$$\frac{dA_3}{dt} = k_6 \cdot C_2 \tag{6}$$

The total amount of the first enzyme will be called E_{t1} and the total amount of the second E_{t2}. The two enzymes appear both in free form and in the form of enzyme-substrate complexes, which gives the conservation equations:

$$E_{t1} = E_1 + C_1 \text{ and} \tag{7}$$

$$E_{t2} = E_2 + C_2 \tag{8}$$

The situation that in the present discussion is of most interest, arises when the concentration of A_2 does not vary with time; the composition of the animal must not change from one moment to the next. This means that $\frac{dA_2}{dt} = 0$ or, in other words, that the rate of formation of A_2 is equal to the rate of disappearance. Assuming steady state conditions for the two enzyme-substrate complexes C_1 and C_2 it can be shown that

$$\frac{k_3 \cdot E_{t1} \cdot A_1}{K_1 + A_1} = \frac{k_6 \cdot E_{t2} \cdot A_2}{K_2 + A_2} \tag{9}$$

where

$$K_1 = \frac{k_2 + k_3}{k_1} \text{ and} \tag{10}$$

$$K_2 = \frac{k_5 + k_6}{k_4} \tag{11}$$

If this constant concentration of A_2 is called A_{2c} and, by rearrangement of equation (9), expressed as a function A_1 we obtain:

$$A_{2c} = \frac{k_3 \cdot E_{t1} \cdot K_2 \cdot A_1}{k_6 \cdot E_{t2} \cdot K_1 + (k_6 \cdot E_{t2} - k_3 \cdot E_{t1}) \cdot A_1} \tag{12}$$

The products $k_3 \cdot E_{11} \cdot$ and $k_6 \cdot E_{12}$ correspond to the maximal rates of formation of A_2 and A_3, *e. g.* the rates obtained when C_1 and C_2 have their maximal values. This occurs when the enzymes are saturated with substrate (E_1 and $E_2 = 0$ in equation (7) and (8)). If these maximal rates are called V_1 and V_2 equation (12) can be written in a slightly more condensed form:

$$A_{2c} = \frac{V_1 \cdot K_2 \cdot A_1}{V_2 \cdot K_1 + (V_2 - V_1) \cdot A_1} \tag{13}$$

It can be seen, that the relation between A_{2c} and A_1 is not straightforward. If A_1 is small, A_{2c} is approximately proportional to A_1, but as A_1 increases towards greater values, A_{2c} gradually becomes independent of A_1. The value of A_{2c} is furthermore dependent on a number of velocity constants and also on the concentrations of the enzymes E_1 and E_2. The last fact is especially noteworthy as enzyme concentrations are known to vary markedly from one animal species to another and even from one individual to an other within a species (WILLIAMS 1956).

An analysis of an organism's content of an intermediate (A_2) does therefore not by itself give any information about the amount of the nutrient (A_1) that is required to maintain a constant level of the intermediate. It follows that if two amino acids, let us call them A_1 and B_1, in an organism or a tissue were going through reaction series of the type shown in equation (1), the ratio $\frac{A_1}{B_1}$ would not be predictable from the ratio $\frac{A_{2c}}{B_{2c}}$ obtained on analysis.

There is reason to assume that in most instances the substrate concentrations occurring within cells are small compared to the saturation values (KREBS 1957, DIXON and WEBB 1958, REINER 1959). The term $(V_2 - V_1) \cdot A_1$ in the denominator of equation (13) may therefore be negligeable compared to $V_2 \cdot K_1$. (This would be true also if V_2 and V_1 had nearly the same numerical value, a situation which seems, however, more unlikely). Under such conditions equation (13) would read

$$A_{2c} = \frac{V_1 \cdot K_2}{V_2 \cdot K_1} \cdot A_1 \tag{14}$$

Similarly the relation between B_{2c} and B_1 could be written

$$B_{2c} = \frac{V'_1 \cdot K'_2}{V'_2 \cdot K'_1} \cdot B_1 \tag{15}$$

and

$$\frac{A_{2c}}{B_{2c}} = \frac{V_1 \cdot K_2}{V_2 \cdot K_1} \cdot \frac{V'_2 \cdot K'_1}{V'_1 \cdot K'_2} \cdot \frac{A_1}{B_1} \tag{16}$$

According to the last equation, the ratio of the concentrations of two intermediates is proportional to the ratio of the concentrations of the substances from which they are formed. But the two ratios are not identical unless

$$\frac{V_1 \cdot K_2 \cdot V'_2 \cdot K'_1}{V_2 \cdot K_1 \cdot V'_1 \cdot K'_2} = 1 \tag{17}$$

Suppose that on analysis the ratio $\frac{A_{2c}}{B_{2c}}$ was found to be 2. If the fraction of constants were $\frac{1}{2}$, the ratio $\frac{A_1}{B_1}$ would be 4.

It should be strongly emphasized that the discussion has been based on a model system (equation (1)) which is far too simple to describe adequately the metabolism of amino acids in the body. We have ignored, for instance, that the amino acids must be transported in one way or another through cell membranes before taking part in the metabolism. The mechanism for this process is not clear, but it probably involves the interaction of the acid with "carriers". In the cell it is more probable that the amino acid will participate in a number of reversible bimolecular reactions rather than in an irreversible monomolecular reaction as stated in equation (1). An example of a reversible bimolecular reaction in which amino acids take part is their activation with adenosine triphosphate, a process that may be a prerequisite for protein synthesis (*cf.* CHANTRENNE 1958). It is not known whether this and other reactions leading to the incorporation of amino acids into proteins are "ordered" or "random" bimolecular reactions. In cases where the latter is true, the reaction rates will depend both on the first and the second power of the substrate concentrations (REINER 1959), thus making the ratio $\frac{A_{2c}}{B_{2c}}$ dependent on A_1 and B_1 in a complex fashion. Bimolecular reactions would in any case introduce more variables on which this ratio would depend. Even for amino acids like threonine or lysine that normally seem to serve no other purpose than as building blocks of proteins, a number of further complications can be conceived. The coupling of protein anabolism (and catabolism) to energy yielding processes, the influence of transit times and the size and composition of the metabolic pool of nitrogen, the fact that the enzymes participating in protein synthesis are themselves proteins, the effect of structural relationships in the derivation of rate equations, the dependence of protein synthesis on nucleic acid synthesis, and other phenomena have all been left out of consideration here. The lack of knowledge of how amino acids are put together to form proteins makes it impossible at present to account for these factors. Therefore, the kinetic speculations outlined above merely serve to emphasize the hazard of drawing conclusions from only the concentration of an intermediate in a reaction sequence.

It is of interest in this connection to mention the kinetic aspects of protein synthesis that have been put forward by STEINBERG, VAUGHAN and ANFINSEN (1956) to account for the nonuniform protein labelling observed by them in experiments with radioactive amino acids. Assuming a stepwise synthesis of a protein via intermediate conjugates of the amino acids in the metabolic pool, they show that the radioactivity of an amino acid in the protein (A_2) depends on the radioactivity of the same acid in the pool (A_1) in the following manner

$$A_2 = k_{1a} \cdot A_1 \left(t + \frac{1}{k_{2a}} \cdot (e^{-k_{2a} \cdot t} - 1)\right) \tag{18}$$

In this formula k_{1a} and k_{2a} are rate constants and t is the time. If t is sufficiently large, A_2 would be approximately equal to $k_{1a} \cdot A_1 \cdot t$. The same relation would hold for another

amino acid B and the ratio $\frac{A_2}{B_2}$ could be written

$$\frac{A_2}{B_2} = \frac{k_{1a}}{k_{1b}} \cdot \frac{A_1}{B_1} \tag{19}$$

an expression resembling (16).

Discussion

Each amino acid takes part in the formation not only of one but a large number of proteins in the organism. It is known that all proteins in the body are not metabolized at the same rate (*cf.* SCHÖNHEIMER 1949, FRIEDBERG, TARVER and GREENBERG 1948). It is by no means certain, that the proteins having the fastest turnover rate and therefore those which we daily have to replace and repair, have the same amino acid composition as the entire animal or as muscle proteins as a whole. Even if the principle of the carcass analysis method were correct, it would thus be dangerous to use the analytical values of the amino acid composition of the whole animal or the whole muscle for the estimation of amino acid requirements.

When amino acids are set free in the body due to protein catabolism they mix with the amino acids of the metabolic pools which contain amino acids also of dietary origin. The amino acids of these pools are either used for protein synthesis or excreted unaltered or in a modified form. A slow breakdown and/or excretion of an amino acid derived from the catabolism of a tissue protein would mean that the amino acid could be reused for protein synthesis to a larger extent than an amino acid that is rapidly disposed of. Attention should be drawn to the fact that the renal clearance of amino acids varies markedly from one amino acid to another and in a fashion that bears no obvious resemblance to the amino acid composition of the entire animal (WRIGHT 1948, FOWLER *et al.* 1957, WILLIAMS 1959).

Some essential amino acids, *e. g.* methionine, tryptophan, phenylalanine and arginine (essential for rats, dogs and chicks) have other functions in the organism besides forming proteins. Methionine, for instance, participates in the formation of creatine, adrenaline, choline, N-methylnicotinamide, spermidine and other compounds, and tryptophan can give rise to niacin and 5-hydroxytryptamine (*cf.* FRUTON and SIMMONDS 1958). It is usual to express amino acid requirements by stating how many times they exceed the requirement of tryptophan. This is, of course, not a satisfactory method, since some of the tryptophan in the diet is used for purposes other than protein synthesis, and this to an extent that depends on the level in the diet of nutritional factors other than amino acids, *e. g.* niacin and vitamin B_6. Some animal species also convert tryptophan to niacin much more efficiently than others.

Table I. Ranges of daily requirements of amino acids for men and women, compiled by R. J. Williams (1959). The requirements cited were determined by nitrogen balance studies

Amino acid	Low mg/day	High mg/day	High/Low
Tryptophan	82	250	3.0
Valine	375	800	2.1
Phenylalanine	420	1,100	2.6
Lysine	400	1,600	4.0
Methionine	[1]175	1,100	6.3
Threonine	103	500	4.9
Leucine	170	1,100	6.5
Isoleucine	250	700	2.8

[1] Only 75 mg was methionine, the rest was cystine.

It is also of significance in a discussion of the carcass analysis method to recall that higher animals have a limited ability to synthesize some essential amino acids, and that certain amino acids have a sparing action on others. Methionine can be synthesized from homocysteine by means of transmethylation from betaine or by *de novo* synthesis of the methyl group. It can also be formed from the corresponding α-keto acid by transamination. Furthermore, cystine can replace a large amount of the daily requirement of methionine. The degree to which such reactions occur varies from one animal species to another and is also determined by the presence in the diet of vitamin B_{12}, folic acid, choline and other factors.

The carcass analysis method is not by itself applicable to the determination of amino acid requirements. First, the method does not give any information about *which* amino acids are indispensable and which are not. Secondly, it is necessary to know the need for *one* of the essential amino acids before the requirements of the others can be calculated. An error that may occur in the determination of the body's need for the reference amino acid will be passed on to all the others.

The ratios of the *concentrations* of amino acids in the human body do not vary significantly from one individual to another. If the carcass method were valid, one should therefore expect the variation in the *requirements* of the different amino acids to be equally small. This is not the case. Two individuals with the same minimal requirement for one amino acid can have greatly different requirements for another amino acid (*cf.* Rose 1957, Leverton 1959). This fact is illustrated in Table I, compiled by Williams (1959), which shows that individual requirements of an amino acid can vary by factors of over 6. Similar variations have been observed also for growing animals (Tappan *et al.*

1952). How such large differences can occur is suggested by the influence of enzyme concentrations on the relation between A_1 and A_2 in formula (12) and the fact that activities of enzymes are — as has already been mentioned — known to vary markedly from one individual to another (WILLIAMS 1956). Enzyme activities also depend on the nutritional status of a given individual (WATERLOW 1959).

The amino acid composition of higher animals does not appear to vary with age (WILLIAMS *et al.* 1954). Although critical evidence for this seems to be lacking in the human, it appears reasonable to assume that it holds true also for this species. It should follow — according to the carcass analysis method — that the ratios of the amino acid requirements for growing children should be identical with the ratios of the concentrations of the amino acids in the adult. This argument has been upheld in particular by ALBANESE (1955, 1959), who has provided evidence that the lysine: tryptophan (L: T) ratio in the diet of infants is optimal at approximately 6.3: 1, which is the L: T-ratio of muscle proteins. The daily lysine requirement of infants is estimated by him to be 170—200 mg/kg body weight and the tryptophan requirement 30 mg/kg. HOLT and SNYDERMAN (1956) seem to accept the same figure for tryptophan but their experiments with infants indicate a daily average lysine requirement of approximately 90 mg/kg. This gives the same average L: T-ratio of 3: 1 for the growth of infants as for the nitrogen balance in adults (*cf.* ROSE 1957; LEVERTON 1959), a ratio that is, however, only half that predicted by the carcass analysis procedure. Both ALBANESE (1959) and HOLT and SNYDERMAN (1956) have referred to body composition data while scrutinizing each others findings. It seems doubtful if this is a reliable basis for discussion.

Various authors have repeatedly emphasized that the amino acid composition of different animals is practically the same, yet the average ratios of the amino acid requirements are not the same for the growth (or nitrogen balance) of the rat, chick, pig or human (*cf.* WILLIAMS *et al.* 1954, ROSE 1957, HOLT and SNYDERMAN 1956, LEVERTON 1959). The rat, for instance, needs less leucine in relation to lysine than the human, although in the rat the ratio of leucine to lysine has actually been reported to be somewhat higher than in the human. Similar situations could be mentioned for other amino acids and other animal species. Also the attempts to calculate correlation coefficients between the amino acid content of various proteins in relation to muscle protein and the biological values of the same proteins (MITCHELL 1959, BOCOBO *et al.* 1952) have, in the author's opinion, given inconsistent results. One can, of course, argue that neither the amino acid analyses nor the estimations of requirements are as yet accurate enough to justify criticism of the carcass analysis method to be based on such failures, but it appears more likely that the reason for the discrepancies is of a fundamental nature. Further work — both theoretical and experimental — is needed to clarify the situation and to define the applicability of the method.

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Further Studies on Pigment Migration and Sensitivity Changes in the Compound Eye of Nocturnal Insects

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It has been shown that the dark adaptation in noctuid moths proceeds in two phases (Bernhard and Ottoson, 1959, 1960 a) and comparative histological studies have brought evidence that the increase in sensitivity during the second phase is mediated by the outward movement of the retinal pigment (Bernhard and Ottoson 1959, 1960 b).

Continued electrophysiological and histological studies on the relationship between the positional changes of the retinal pigment and the sensitivity of the eye have now been carried out on the isolated eye of the noctuid moth *Cerapteryx graminis*. In 21 eyes the sensitivity changes were measured electrophysiologically (see Bernhard and Ottoson 1960 a) during dark adaptation and during adaptation to light of different intensities after exposure of the eye to bright light (pre-adapting illumination 1—3 min; relative intensity — 1). Fig. 1 A shows the typical discontinuous dark adaptation curve, the second phase of which starts about 9 min after cessation of the bright light. Curve 1 B, showing the sensitivity changes during the exposure of the eye to an adapting light with a relative brightness of — 3.4, has no corresponding second phase. The lack of this phase is not due to impairment of the eye since there is a sudden fall in the curve as soon as the adapting dim light is shut off (at vertical arrow). Curves C and D obtained with adapting lights with lower light intensities (— 4.6 respectively — 6.4) show a second phase which is more pronounced at the lowest intensity of the adapting light. As expected, cessation of the dim light (vertical arrow), is followed by a further decrease in the threshold. All curves obtained with adapting light intensities above — 4.6 (between — 2.2 and 4.3) showed no second phase, whereas 14 curves out of 16 obtained with adapting intensities between — 4.6 and — 7, showed a second phase which in general was greater at lower intensities. Whereas the second phase appears with a long latency during dark adaptation after bright pre-adapting light (curve A), the further fall in the curves appears without appreciable delay, when darkness follows exposure to light of lower intensities (curves B, C, D).

Dark adapted eyes as well as eyes which had been adapted for 20 min to light of different intensities, ranging from — 3.4 to — 7, were fixed in Bouin-Duboscq-Brasil's solution and stained with haemalum-eosin. There was no

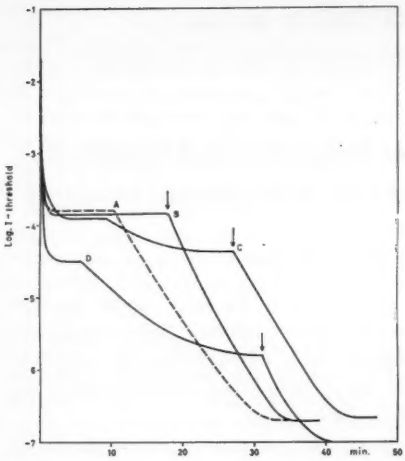

Fig. 1. Dark-adaptation curve of *Cerapteryx graminis* (A) and curves showing the relation between threshold intensities and time of adaptation to light of different intensities (B, C and D; for further description see text).

pigment retraction in the eyes which had been exposed to light intensities above — 4.6, whereas a retraction of varying degree was seen in the eyes which had been exposed to intensities between — 4.6 and — 6, maximal retraction being found in most eyes exposed to intensities below — 6.

The facts 1) that neither the second phase of the adaptation curve nor the pigment migration were obtained when the eyes were exposed to light with intensities above a certain value (— 4.6), whereas both the second phase and the pigment migration were found at light intensities below this value and 2) that there is a relation between the magnitude of fall of threshold during the second phase and the degree of pigment retraction with dim light of different intensities, give further evidence that the second phase of the adaptation curve, is correlated to the outward movement of the pigment. Taking this conclusion into consideration, the curves in Fig. 1 would show that the pigment retraction starts after a long latency of several minutes after exposure of the eye to bright light, whereas the retraction begins without appreciable delay when the eye has been exposed to lower light intensities.

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References

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ERRATA

p. 19 line 4 from below: 10^3 c. p. s. should be replaced by 10^4 c. p. s.

p. 63 line 3 from below: see footnote p. 000 should be replaced by see footnote p. 62.

p. 77 BOEMINGHAUS & KOCHMANN, last column: 0.15 should be replaced by 0.015 and 0.23 should be replaced by 0.023.

p. 77 BENNETT et al., column 7: 0.1–0.15 mM should be replaced by 1–1.5 mM.

p. 135 equation (1): exponent c should be replaced by s;
line 14 from below: c should be replaced by s.

p. 143 equation (8) should be replaced by $s = 7 \times 10^{-7}$ sec^{-1};
last line: cm$^{-3} \times$ sec^{-1} should be replaced by sec^{-1}.

p. 144 line 8: 1.5 should be replaced by 2.1;
equation (10): 7.5 should be replaced by 5.3.

ERRATUM

Vol. 51. Fasc. 2—3, page 177, line 8: 2 mg, read 2 μg.